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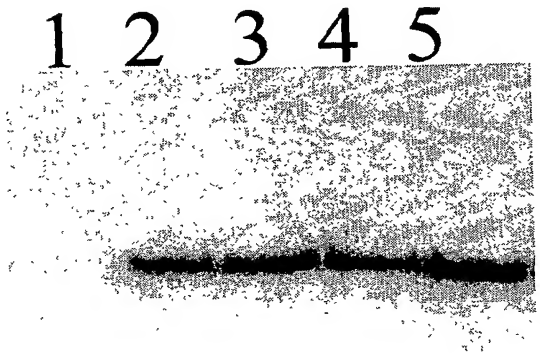
(54) Title: ANTIBODIES AND METHODS FOR GENERATING GENETICALLY ALTERED ANTIBODIES WITH HIGH AFFINITY



← Ig heavy chain

(57) Abstract: Dominant negative alleles of human mismatch repair genes can be used to generate hypermutable cells and organisms. By introducing these genes into cells and transgenic animals, new cell lines and animal varieties with novel and useful properties can be prepared more efficiently than by relying on the natural rate of mutation. These methods are useful for generating genetic diversity within immunoglobulins genes directed against an antigen of interest to produce altered antibodies with enhanced biochemical activity. Moreover, these methods are useful for generating antibody-producing cells with increased level of antibody production. The invention also provides methods for increasing the affinity of monoclonal antibodies and monoclonal antibodies with increased affinity.

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ANTIBODIES AND METHODS FOR GENERATING GENETICALLY ALTERED ANTIBODIES WITH HIGH AFFINITY

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Application No. 10/243,130, filed September 13, 2002, which is a continuation-in-part of U.S. Serial No. 09/707,468, filed November 7, 2000, the disclosures of which are hereby incorporated by reference in their entirety.

TECHNICAL FIELD OF THE INVENTION

[0002] The invention is related to the area of antibody maturation and cellular production. In particular, it is related to the field of mutagenesis.

BACKGROUND OF THE INVENTION

[0003] The use of antibodies to block the activity of foreign and/or endogenous polypeptides provides an effective and selective strategy for treating the underlying cause of disease. In particular is the use of monoclonal antibodies (MAb) as effective therapeutics such as the FDA approved ReoPro (Glaser, V. (1996) Can ReoPro repolish tarnished monoclonal therapeutics? *Nat. Biotechnol.* 14:1216-1217), an anti-platelet MAb from Centocor; Herceptin (Weiner, L.M. (1999) Monoclonal antibody therapy of cancer. *Semin. Oncol.* 26:43-51), an anti-Her2/neu MAb from Genentech; and Synagis (Saez-Llorens, X.E., *et al.* (1998) Safety and pharmacokinetics of an intramuscular humanized monoclonal antibody to respiratory syncytial virus in premature infants and infants with bronchopulmonary dysplasia.

Pediat. Infect. Dis. J. 17:787-791), an anti-respiratory syncytial virus MAb produced by Medimmune.

[0004] Standard methods for generating MAbs against candidate protein targets are known by those skilled in the art. Briefly, rodents such as mice or rats are injected with a purified antigen in the presence of adjuvant to generate an immune response (Shield, C.F., *et al.* (1996) A cost-effective analysis of OKT3 induction therapy in cadaveric kidney transplantation. *Am. J. Kidney Dis.* 27:855-864). Rodents with positive immune sera are sacrificed and splenocytes are isolated. Isolated splenocytes are fused to melanomas to produce immortalized cell lines that are then screened for antibody production. Positive lines are isolated and characterized for antibody production. The direct use of rodent MAbs as human therapeutic agents were confounded by the fact that human anti-rodent antibody (HARA) responses occurred in a significant number of patients treated with the rodent-derived antibody (Khazaeli, M.B., *et al.*, (1994) Human immune response to monoclonal antibodies. *J. Immunother.* 15:42-52). In order to circumvent the problem of HARA, the grafting of the complementarity determining regions (CDRs), which are the critical motifs found within the heavy and light chain variable regions of the immunoglobulin (Ig) subunits making up the antigen binding domain, onto a human antibody backbone found these chimeric molecules are able to retain their binding activity to antigen while lacking the HARA response (Emery, S.C., and Harris, W.J. "Strategies for humanizing antibodies" In: ANTIBODY ENGINEERING C.A.K. Borrebaeck (Ed.) Oxford University Press, N.Y. 1995. pp. 159-183. A common problem that exists during the "humanization" of rodent-derived MAbs (referred to hereon as HAb) is the loss of binding affinity due to conformational changes in the 3 dimensional structure of the CDR domain upon grafting onto the human Ig backbone (U.S. Patent No. 5,530,101 to Queen *et al.*). To overcome this problem, additional HAb vectors are usually needed to be engineeredby inserting or deleting additional amino acid residues within the framework region and/or within the CDR coding region itself in order to recreate high affinity HAbs (U.S. Patent No. 5,530,101 to Queen *et al.*). This process is a very time consuming procedure that involves the use of expensive computer modeling programs to predict changes that may lead to a high affinity HAb. In some instances the

affinity of the HAb is never restored to that of the MAb, rendering them of little therapeutic use.

[0005] Another problem that exists in antibody engineering is the generation of stable, high yielding producer cell lines that is required for manufacturing of the molecule for clinical materials. Several strategies have been adopted in standard practice by those skilled in the art to circumvent this problem. One method is the use of Chinese Hamster Ovary (CHO) cells transfected with exogenous Ig fusion genes containing the grafted human light and heavy chains to produce whole antibodies or single chain antibodies, which are a chimeric molecule containing both light and heavy chains that form an antigen-binding polypeptide (Reff, M.E. (1993) High-level production of recombinant immunoglobulins in mammalian cells. *Curr. Opin. Biotechnol.* 4:573-576). Another method employs the use of human lymphocytes derived from transgenic mice containing a human grafted immune system or transgenic mice containing a human Ig gene repertoire. Yet another method employs the use of monkeys to produce primate MAbs, which have been reported to lack a human anti-monkey response (Neuberger, M., and Gruggermann, M. (1997) Monoclonal antibodies. Mice perform a human repertoire. *Nature* 386:25-26). In all cases, the generation of a cell line that is capable of generating sufficient amounts of high affinity antibody poses a major limitation for producing sufficient materials for clinical studies. Because of these limitations, the utility of other recombinant systems such as plants are currently being explored as systems that will lead to the stable, high-level production of humanized antibodies (Fiedler, U., and Conrad, U. (1995) High-level production and long-term storage of engineered antibodies in transgenic tobacco seeds. *Bio/Technology* 13:1090-1093).

[0006] A method for generating diverse antibody sequences within the variable domain that results in HAbs and MAbs with high binding affinities to antigens would be useful for the creation of more potent therapeutic and diagnostic reagents respectively. Moreover, the generation of randomly altered nucleotide and polypeptide residues throughout an entire antibody molecule will result in new reagents that are less antigenic and/or have beneficial pharmacokinetic properties. The invention described herein is directed to the use of random genetic mutation throughout an antibody structure *in vivo* by blocking the endogenous mismatch repair (MMR) activity of a host cell producing immunoglobulins that encode

biochemically active antibodies. The invention also relates to methods for repeated *in vivo* genetic alterations and selection for antibodies with enhanced binding and pharmacokinetic profiles.

[0007] In addition, the ability to develop genetically altered host cells that are capable of secreting increased amounts of antibody will also provide a valuable method for creating cell hosts for product development. The invention described herein is directed to the creation of genetically altered cell hosts with increased antibody production via the blockade of MMR.

[0008] The invention facilitates the generation of high affinity antibodies and the production of cell lines with elevated levels of antibody production. Other advantages of the present invention are described in the examples and figures described herein.

SUMMARY OF THE INVENTION

[0009] The invention provides methods for generating genetically altered antibodies (including single chain molecules) and antibody producing cell hosts *in vitro* and *in vivo*, whereby the antibody possess a desired biochemical property(s), such as, but not limited to, increased antigen binding, increased gene expression, and/or enhanced extracellular secretion by the cell host. One method for identifying antibodies with increased binding activity or cells with increased antibody production is through the screening of MMR defective antibody producing cell clones that produce molecules with enhanced binding properties or clones that have been genetically altered to produce enhanced amounts of antibody product.

[0010] The antibody producing cells suitable for use in the invention include, but are not limited to rodent, primate, or human hybridomas or lymphoblastoids; mammalian cells transfected and expressing exogenous Ig subunits or chimeric single chain molecules; plant cells, yeast or bacteria transfected and expressing exogenous Ig subunits or chimeric single chain molecules.

[0011] Thus, the invention provides methods for making hypermutable antibody-producing cells by introducing a polynucleotide comprising a dominant negative allele of a mismatch repair gene into cells that are capable of producing antibodies. The cells that are capable of producing antibodies include cells that naturally produce antibodies, and cells that are engineered to produce antibodies through the introduction of immunoglobulin encoding

sequences. Conveniently, the introduction of polynucleotide sequences into cells is accomplished by transfection.

[0012] The invention also provides methods of making hypermutable antibody producing cells by introducing a dominant negative mismatch repair (MMR) gene such as *PMS2* (preferably human *PMS2*), *MLH1*, *PMS1*, *MSH2*, or *MSH2* into cells that are capable of producing antibodies. The dominant negative allele of a mismatch repair gene may be a truncation mutation of a mismatch repair gene (preferably a truncation mutation at codon 134, or a thymidine at nucleotide 424 of wild-type *PMS2*). The invention also provides methods in which mismatch repair gene activity is suppressed. This may be accomplished, for example, using antisense molecules directed against the mismatch repair gene or transcripts.

[0013] Other embodiments of the invention provide methods for making hypermutable antibody-producing cells by introducing a polynucleotide comprising a dominant negative allele of a mismatch repair gene into fertilized eggs of animals. These methods may also include subsequently implanting the eggs into pseudo-pregnant females whereby the fertilized eggs develop into a mature transgenic animal. The mismatch repair genes may include, for example, *PMS2* (preferably human *PMS2*), *MLH1*, *PMS1*, *MSH2*, or *MSH2*. The dominant negative allele of a mismatch repair gene may be a truncation mutation of a mismatch repair gene (preferably a truncation mutation at codon 134, or a thymidine at nucleotide 424 of wild-type *PMS2*).

[0014] The invention further provides homogeneous compositions of cultured, hypermutable, mammalian cells that are capable of producing antibodies and contain a dominant negative allele of a mismatch repair gene. The mismatch repair genes may include, for example, *PMS2* (preferably human *PMS2*), *MLH1*, *PMS1*, *MSH2*, or *MSH2*. The dominant negative allele of a mismatch repair gene may be a truncation mutation of a mismatch repair gene (preferably a truncation mutation at codon 134, or a thymidine at nucleotide 424 of wild-type *PMS2*). The cells of the culture may contain *PMS2*, (preferably human *PMS2*), *MLH1*, or *PMS1*; or express a human *mutL* homolog, or the first 133 amino acids of hPMS2.

[0015] The invention further provides methods for generating a mutation in an immunoglobulin gene of interest by culturing an immunoglobulin producing cell selected for an immunoglobulin of interest wherein the cell contains a dominant negative allele of a

mismatch repair gene. The properties of the immunoglobulin produced from the cells can be assayed to ascertain whether the immunoglobulin gene harbors a mutation. The assay may be directed to analyzing a polynucleotide encoding the immunoglobulin, or may be directed to the immunoglobulin polypeptide itself.

[0016] The invention also provides methods for generating a mutation in a gene affecting antibody production in an antibody-producing cell by culturing the cell expressing a dominant negative allele of a mismatch repair gene, and testing the cell to determine whether the cell harbors mutations within the gene of interest, such that a new biochemical feature (*e.g.*, over-expression and/or secretion of immunoglobulin products) is generated. The testing may include analysis of the steady state expression of the immunoglobulin gene of interest, and/or analysis of the amount of secreted protein encoded by the immunoglobulin gene of interest. The invention also embraces prokaryotic and eukaryotic transgenic cells made by this process, including cells from rodents, non-human primates and humans.

[0017] Other aspects of the invention encompass methods of reversibly altering the hypermutability of an antibody producing cell, in which an inducible vector containing a dominant negative allele of a mismatch repair gene operably linked to an inducible promoter is introduced into an antibody-producing cell. The cell is treated with an inducing agent to express the dominant negative mismatch repair gene (which can be *PMS2* (preferably human *PMS2*), *MLH1*, or *PMS1*). Alternatively, the cell may be induced to express a human *mutL* homolog or the first 133 amino acids of hPMS2. In another embodiment, the cells may be rendered capable of producing antibodies by co-transfecting a preselected immunoglobulin gene of interest. The immunoglobulin genes of the hypermutable cells, or the proteins produced by these methods may be analyzed for desired properties, and induction may be stopped such that the genetic stability of the host cell is restored.

[0018] The invention also embraces methods of producing genetically altered antibodies by transfecting a polynucleotide encoding an immunoglobulin protein into a cell containing a dominant negative mismatch repair gene (either naturally or in which the dominant negative mismatch repair gene was introduced into the cell), culturing the cell to allow the immunoglobulin gene to become mutated and produce a mutant immunoglobulin, screening for a desirable property of said mutant immunoglobulin protein, isolating the polynucleotide

molecule encoding the selected mutant immunoglobulin possessing the desired property, and transfecting said mutant polynucleotide into a genetically stable cell, such that the mutant antibody is consistently produced without further genetic alteration. The dominant negative mismatch repair gene may be *PMS2* (preferably human *PMS2*), *MLH1*, or *PMS1*. Alternatively, the cell may express a human *mutL* homolog or the first 133 amino acids of hPMS2.

[0019] The invention further provides methods for generating genetically altered cell lines that express enhanced amounts of an antigen binding polypeptide. These antigen-binding polypeptides may be, for example, immunoglobulins. The methods of the invention also include methods for generating genetically altered cell lines that secrete enhanced amounts of an antigen binding polypeptide. The cell lines are rendered hypermutable by dominant negative mismatch repair genes that provide an enhanced rate of genetic hypermutation in a cell producing antigen-binding polypeptides such as antibodies. Such cells include, but are not limited to hybridomas. Expression of enhanced amounts of antigen binding polypeptides may be through enhanced transcription or translation of the polynucleotides encoding the antigen binding polypeptides, or through the enhanced secretion of the antigen binding polypeptides, for example.

[0020] Methods are also provided for creating genetically altered antibodies *in vivo* by blocking the MMR activity of the cell host, or by transfecting genes encoding for immunoglobulin in a MMR defective cell host.

[0021] Antibodies with increased binding properties to an antigen due to genetic changes within the variable domain are provided in methods of the invention that block endogenous MMR of the cell host. Antibodies with increased binding properties to an antigen due to genetic changes within the CDR regions within the light and/or heavy chains are also provided in methods of the invention that block endogenous MMR of the cell host.

[0022] The invention provides methods of creating genetically altered antibodies in MMR defective Ab producer cell lines with enhanced pharmacokinetic properties in host organisms including but not limited to rodents, primates, and man.

[0023] These and other aspects of the invention are provided by one or more of the embodiments described below. In one embodiment of the invention, a method for making an

antibody producing cell line hypermutable is provided. A polynucleotide encoding a dominant negative allele of a MMR gene is introduced into an antibody-producing cell. The cell becomes hypermutable as a result of the introduction of the gene.

[0024] In another embodiment of the invention, a method is provided for introducing a mutation into an endogenous gene encoding for an immunoglobulin polypeptide or a single chain antibody. A polynucleotide encoding a dominant negative allele of a MMR gene is introduced into a cell. The cell becomes hypermutable as a result of the introduction and expression of the MMR gene allele. The cell further comprises an immunoglobulin gene of interest. The cell is grown and tested to determine whether the gene encoding for an immunoglobulin or a single chain antibody of interest harbors a mutation. In another aspect of the invention, the gene encoding the mutated immunoglobulin polypeptide or single chain antibody may be isolated and expressed in a genetically stable cell. In a preferred embodiment, the mutated antibody is screened for at least one desirable property such as, but not limited to, enhanced binding characteristics.

[0025] In another embodiment of the invention, a gene or set of genes encoding for Ig light and heavy chains or a combination therein are introduced into a mammalian cell host that is MMR defective. The cell is grown, and clones are analyzed for antibodies with enhanced binding characteristics.

[0026] In another embodiment of the invention, a method will be provided for producing new phenotypes of a cell. A polynucleotide encoding a dominant negative allele of a MMR gene is introduced into a cell. The cell becomes hypermutable as a result of the introduction of the gene. The cell is grown. The cell is tested for the expression of new phenotypes where the phenotype is enhanced secretion of a polypeptide.

[0027] The invention also provides antibodies having increased affinity for antigen comprising immunoglobulin molecules wherein a substitution has been made for at least one amino acid in the variable domain of the heavy and/or light chain. In some embodiments, the substitution is in a position wherein the parental amino acid in that position is an amino acid with a non-polar side chain. In some embodiments the parental amino acid is substituted with a different amino acid that has a non-polar side chain. In other embodiments, the parental amino acid is replaced with a proline or hydroxyproline. In some embodiments, the

substitution(s) are made in the framework regions of the heavy and/or light chain variable domains. In some embodiments, the substitution(s) are made within the first framework region of the heavy chain. In some embodiments, the substitution(s) are made within the second framework region of the light chain. In some embodiments, the substitutions are made within the first framework region of the heavy chain and the second framework region of the light chain. In some embodiments, a substitution is made at position 6 of the first framework region of the heavy chain as shown in SEQ ID NO:18. In some embodiments a substitution is made at position 22 of the second framework region of the light chain as shown in SEQ ID NO:21. For the specific position mutations, in some embodiments the amino acid substitution is a proline or hydroxyproline.

[0028] The invention also provides methods for increasing the affinity of an antibody for an antigen comprising substituting an amino acid within the variable domain of the heavy or light chain of the subject antibody with another amino acid having a non-polar side chain. In some embodiments, a proline is substituted for the original amino acid at the position. In some embodiments, proline is used to substitute for another amino acid having a non-polar side chain. In some embodiments alanine and/or leucine is replaced by proline. In certain embodiments, the amino acid in position 6 of the first framework region of the heavy chain of the antibody as shown in SEQ ID NO:18 is replaced with a proline. In other embodiments, the amino acid in position 22 of the second framework region of the light chain variable domain as shown in SEQ ID NO:21 is replaced with proline. The invention also provides antibodies produced by these methods.

[0029] The antibodies produced in the invention may be made using the process of the invention wherein a dominant negative allele of a mismatch repair gene is introduced into the antibody producing cell and the cell becomes hypermutable as described more fully herein. Alternatively, one may disrupt mismatch repair using chemical inhibitors of mismatch repair, such as using anthracene and/or its derivatives as described in PCT Publication No. WO 02/054856, published July 18, 2002, which is specifically incorporated herein in its entirety. The cells treated with the chemicals that disrupt mismatch repair or which express a dominant-negative mismatch repair gene become hypermutable. The antibodies produced by the hypermutable cells are screened for increased affinity, and those antibodies comprising

the amino acid substitutions described above display increased affinity for antigen. The cells producing the antibodies which have the increased affinity and the molecular characteristics described herein may be rendered genetically stable again by withdrawing the chemical inhibitor, or by rendering the cells genetically stable through the inactivation of the expression of the dominant negative allele. For example, a dominant negative allele that is under the control of an inducible promoter may be inactivated by withdrawing the inducer. Alternatively, the dominant negative allele may be knocked out, or a CRE-LOX expression system may be used whereby the dominant negative allele is spliced from the genome once the cells containing a genetically diverse immunoglobulin have been established.

[0030] In other embodiments, one of skill in the art may use any known method of introducing mutations into proteins and selecting for antibodies having higher affinity with the amino acid substitutions described above. Methods of introducing mutations may be random, such as chemical mutagenesis, or may be specific, such as site-directed mutagenesis. Methods for random and specific mutagenesis are well-known in the art and include, but are not limited to, for example, chemical mutagenesis (*e.g.*, using such chemicals as methane sulfonate, dimethyl sulfonate, O6-methyl benzadine, methylnitrosourea (MNU), and ethylnitrosourea (ENU)); oligonucleotide-mediated site-directed mutagenesis; alanine scanning; and PCR mutagenesis (see, for example, Kunkel *et al.* (1991) *Methods Enzymol.* 204:125-139, site-directed mutagenesis; Crameri *et al.* (1995) *BioTechniques* 18(2):194-196, cassette mutagenesis; and Haught *et al.* (1994) *BioTechniques* 16(1):47-48, restriction selection mutagenesis).

[0031] These and other embodiments of the invention provide the art with methods that can generate enhanced mutability in cells and animals as well as providing cells and animals harboring potentially useful mutations for the large-scale production of high affinity antibodies with beneficial pharmacokinetic profiles.

BRIEF DESCRIPTION OF THE DRAWINGS

[0032] Figure 1. Hybridoma cells stably expressing PMS2 and PMS134 MMR genes. Shown is steady state mRNA expression of MMR genes transfected into a murine hybridoma cell line. Stable expression was found after 3 months of continuous growth. The (–) lanes

represent negative controls where no reverse transcriptase was added, and the (+) lanes represent samples reverse transcribed and PCR amplified for the MMR genes and an internal housekeeping gene as a control.

[0033] Figure 2. Creation of genetically hypermutable hybridoma cells. Dominant negative MMR gene alleles were expressed in cells expressing a MMR-sensitive reporter gene. Dominant negative alleles such as PMS134 and the expression of MMR genes from other species results in antibody producer cells with a hypermutable phenotype that can be used to produce genetically altered immunoglobulin genes with enhanced biochemical features as well as lines with increased Ig expression and/or secretion. Values shown represent the amount of converted CPRG substrate which is reflective of the amount of function β-galactosidase contained within the cell from genetic alterations within the pCAR-OF reporter gene. Higher amounts of β-galactosidase activity reflect a higher mutation rate due to defective MMR.

[0034] Figure 3. Screening method for identifying antibody-producing cells containing antibodies with increased binding activity and/or increased expression/secretion.

[0035] Figure 4. Generation of a genetically altered antibody with an increased binding activity. Shown are ELISA values from 96-well plates, screened for antibodies specific to hIgE. Two clones with a high binding value were found in HB134 cultures.

[0036] Figure 5. Sequence alteration within variable chain of an antibody (a mutation within the light chain variable region in MMR-defective HB134 antibody producer cells). An arrow indicates the nucleotide at which a mutation occurred in a subset of cells from a clone derived from HB134 cells. In Figure 5A, the change results in a Thr to Ser change within the light chain variable region. The coding sequence is in the antisense direction. In Figure 5B, the change results in a Pro to His change within the light chain variable region.

[0037] Figure 6. Generation of MMR-defective clones with enhanced steady state Ig protein levels. A Western blot of heavy chain immunglobulins from HB134 clones with high levels of MAb (>500ngs/ml) within the conditioned medium shows that a subset of clones express higher steady state levels of immunoglobulins (Ig). The H36 cell line was used as a control to measure steady state levels in the parental strain. Lane 1: fibroblast cells (negative control);

Lane 2: H36 cell; Lane 3: HB134 clone with elevated MAb levels; Lane 4: HB134 clone with elevated MAb levels; Lane 5: HB134 clone with elevated MAb levels.

[0038] Methods have been discovered for developing hypermutable antibody-producing cells by taking advantage of the conserved mismatch repair (MMR) process of host cells. Dominant negative alleles of such genes, when introduced into cells or transgenic animals, increase the rate of spontaneous mutations by reducing the effectiveness of DNA repair and thereby render the cells or animals hypermutable. Hypermutable cells or animals can then be utilized to develop new mutations in a gene of interest. Blocking MMR in antibody-producing cells such as but not limited to: hybridomas; mammalian cells transfected with genes encoding for Ig light and heavy chains; mammalian cells transfected with genes encoding for single chain antibodies; eukaryotic cells transfected with Ig genes, can enhance the rate of mutation within these cells leading to clones that have enhanced antibody production and/or cells containing genetically altered antibodies with enhanced biochemical properties such as increased antigen binding. The process of MMR, also called mismatch proofreading, is carried out by protein complexes in cells ranging from bacteria to mammalian cells. A MMR gene is a gene that encodes for one of the proteins of such a mismatch repair complex. Although not wanting to be bound by any particular theory of mechanism of action, a MMR complex is believed to detect distortions of the DNA helix resulting from non-complementary pairing of nucleotide bases. The non-complementary base on the newer DNA strand is excised, and the excised base is replaced with the appropriate base, which is complementary to the older DNA strand. In this way, cells eliminate many mutations that occur as a result of mistakes in DNA replication.

[0039] Dominant negative alleles cause a MMR defective phenotype even in the presence of a wild-type allele in the same cell. An example of a dominant negative allele of a MMR gene is the human gene *hPMS2-134*, which carries a truncating mutation at codon 134 (SEQ ID NO:15). The mutation causes the product of this gene to abnormally terminate at the position of the 134th amino acid, resulting in a shortened polypeptide containing the N-terminal 133 amino acids. Such a mutation causes an increase in the rate of mutations, which accumulate in cells after DNA replication. Expression of a dominant negative allele of a mismatch repair gene results in impairment of mismatch repair activity, even in the presence of the wild-type

allele. Any allele which produces such effect can be used in this invention. Dominant negative alleles of a MMR gene can be obtained from the cells of humans, animals, yeast, bacteria, or other organisms. Such alleles can be identified by screening cells for defective MMR activity. Cells from animals or humans with cancer can be screened for defective mismatch repair. Cells from colon cancer patients may be particularly useful. Genomic DNA, cDNA, or mRNA from any cell encoding a MMR protein can be analyzed for variations from the wild type sequence. Dominant negative alleles of a MMR gene can also be created artificially, for example, by producing variants of the *hPMS2-134* allele or other MMR genes. Various techniques of site-directed mutagenesis can be used. The suitability of such alleles, whether natural or artificial, for use in generating hypermutable cells or animals can be evaluated by testing the mismatch repair activity caused by the allele in the presence of one or more wild-type alleles, to determine if it is a dominant negative allele.

[0040] A cell or an animal into which a dominant negative allele of a mismatch repair gene has been introduced will become hypermutable. This means that the spontaneous mutation rate of such cells or animals is elevated compared to cells or animals without such alleles. The degree of elevation of the spontaneous mutation rate can be at least 2-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, 200-fold, 500-fold, or 1000-fold that of the normal cell or animal. The use of chemical mutagens such as but limited to methane sulfonate, dimethyl sulfonate, O6-methyl benzadine, MNU, ENU, etc. can be used in MMR defective cells to increase the rates an additional 10 to 100 fold that of the MMR deficiency itself.

[0041] According to one aspect of the invention, a polynucleotide encoding for a dominant negative form of a MMR protein is introduced into a cell. The gene can be any dominant negative allele encoding a protein, which is part of a MMR complex, for example, *PMS2, PMS1, MLH1*, or *MSH2*. The dominant negative allele can be naturally occurring or made in the laboratory. The polynucleotide can be in the form of genomic DNA, cDNA, RNA, or a chemically synthesized polynucleotide.

[0042] The polynucleotide can be cloned into an expression vector containing a constitutively active promoter segment (such as but not limited to CMV, SV40, Elongation Factor or LTR sequences) or to inducible promoter sequences such as the steroid inducible pIND vector

(Invitrogen), where the expression of the dominant negative MMR gene can be regulated. The polynucleotide can be introduced into the cell by transfection.

[0043] According to another aspect of the invention, an immunoglobulin (Ig) gene, a set of Ig genes or a chimeric gene containing whole or parts of an Ig gene can be transfected into MMR deficient cell hosts, the cell is grown and screened for clones containing genetically altered Ig genes with new biochemical features. MMR defective cells may be of human, primates, mammals, rodent, plant, yeast or of the prokaryotic kingdom. The mutated gene encoding the Ig with new biochemical features may be isolated from the respective clones and introduced into genetically stable cells (*i.e.*, cells with normal MMR) to provide clones that consistently produce Ig with the new biochemical features. The method of isolating the Ig gene encoding Ig with new biochemical features may be any method known in the art. Introduction of the isolated polynucleotide encoding the Ig with new biochemical features may also be performed using any method known in the art, including, but not limited to transfection of an expression vector containing the polynucleotide encoding the Ig with new biochemical features. As an alternative to transfecting an Ig gene, a set of Ig genes or a chimeric gene containing whole or parts of an Ig gene into an MMR deficient host cell, such Ig genes may be transfected simultaneously with a gene encoding a dominant negative mismatch repair gene into a genetically stable cell to render the cell hypermutable.

[0044] Transfection is any process whereby a polynucleotide is introduced into a cell. The process of transfection can be carried out in a living animal, *e.g.*, using a vector for gene therapy, or it can be carried out *in vitro*, *e.g.*, using a suspension of one or more isolated cells in culture. The cell can be any type of eukaryotic cell, including, for example, cells isolated from humans or other primates, mammals or other vertebrates, invertebrates, and single celled organisms such as protozoa, yeast, or bacteria.

[0045] In general, transfection will be carried out using a suspension of cells, or a single cell, but other methods can also be applied as long as a sufficient fraction of the treated cells or tissue incorporates the polynucleotide so as to allow transfected cells to be grown and utilized. The protein product of the polynucleotide may be transiently or stably expressed in the cell. Techniques for transfection are well known. Available techniques for introducing polynucleotides include but are not limited to electroporation, transduction, cell fusion, the

use of calcium chloride, and packaging of the polynucleotide together with lipid for fusion with the cells of interest. Once a cell has been transfected with the MMR gene, the cell can be grown and reproduced in culture. If the transfection is stable, such that the gene is expressed at a consistent level for many cell generations, then a cell line results.

[0046] An isolated cell is a cell obtained from a tissue of humans or animals by mechanically separating out individual cells and transferring them to a suitable cell culture medium, either with or without pretreatment of the tissue with enzymes, *e.g.*, collagenase or trypsin. Such isolated cells are typically cultured in the absence of other types of cells. Cells selected for the introduction of a dominant negative allele of a mismatch repair gene may be derived from a eukaryotic organism in the form of a primary cell culture or an immortalized cell line, or may be derived from suspensions of single-celled organisms.

[0047] A polynucleotide encoding for a dominant negative form of a MMR protein can be introduced into the genome of an animal by producing a transgenic animal. The animal can be any species for which suitable techniques are available to produce transgenic animals. For example, transgenic animals can be prepared from domestic livestock, *e.g.*, bovine, swine, sheep, goats, horses, etc.; from animals used for the production of recombinant proteins, *e.g.*, bovine, swine, or goats that express a recombinant polypeptide in their milk; or experimental animals for research or product testing, *e.g.*, mice, rats, guinea pigs, hamsters, rabbits, etc. Cell lines that are determined to be MMR defective can then be used as a source for producing genetically altered immunoglobulin genes *in vitro* by introducing whole, intact immunoglobulin genes and/or chimeric genes encoding for single chain antibodies into MMR defective cells from any tissue of the MMR defective animal.

[0048] Once a transfected cell line or a colony of transgenic animals has been produced, it can be used to generate new mutations in one or more gene(s) of interest. A gene of interest can be any gene naturally possessed by the cell line or transgenic animal or introduced into the cell line or transgenic animal. An advantage of using such cells or animals to induce mutations is that the cell or animal need not be exposed to mutagenic chemicals or radiation, which may have secondary harmful effects, both on the object of the exposure and on the workers. However, chemical mutagens may be used in combination with MMR deficiency, which renders such mutagens less toxic due to an undetermined mechanism. Hypermutable

animals can then be bred and selected for those producing genetically variable B-cells that may be isolated and cloned to identify new cell lines that are useful for producing genetically variable cells. Once a new trait is identified, the dominant negative MMR gene allele can be removed by directly knocking out the allele by technologies used by those skilled in the art or by breeding to mates lacking the dominant negative allele to select for offspring with a desired trait and a stable genome. Another alternative is to use a CRE-LOX expression system, whereby the dominant negative allele is spliced from the animal genome once an animal containing a genetically diverse immunoglobulin profile has been established. Yet another alternative is the use of inducible vectors such as the steroid induced pIND (Invitrogen) or pMAM (Clonetech) vectors which express exogenous genes in the presence of corticosteroids.

[0049] Mutations can be detected by analyzing for alterations in the genotype of the cells or animals, for example by examining the sequence of genomic DNA, cDNA, messenger RNA, or amino acids associated with the gene of interest. Mutations can also be detected by screening for the production of antibody titers. A mutant polypeptide can be detected by identifying alterations in electrophoretic mobility, spectroscopic properties, or other physical or structural characteristics of a protein encoded by a mutant gene. One can also screen for altered function of the protein *in situ*, in isolated form, or in model systems. One can screen for alteration of any property of the cell or animal associated with the function of the gene of interest, such as but not limited to Ig secretion.

[0050] Examples of nucleic acid sequences encoding mismatch repair proteins include, but are not limited to the following: mouse *PMS2* (SEQ ID NO:6); human *PMS2* (SEQ ID NO:8); human *PMS1* (SEQ ID NO:10) human *MSH2* (SEQ ID NO:12); human *MLH1* (SEQ ID NO:14); and human *PMS2-134* (SEQ ID NO:16). The corresponding amino acid sequences are: mouse *PMS2* (SEQ ID NO:5); human *PMS2* (SEQ ID NO:7); human *PMS1* (SEQ ID NO:9) human *MSH2* (SEQ ID NO:11); human *MLH1* (SEQ ID NO:13); and human *PMS2-134* (SEQ ID NO:15).

[0051] Mutant antibodies showing increased affinity for antigen were sequenced and compared to the sequence of the wild-type (WT) H36 parental antibody. It has been discovered that alterations of amino acids to proline has the effect of increasing affinity for

antigen when introduced into the variable region of either the light chain or heavy chain of the immunoglobulin molecule. While not wishing to be bound by any particular theory of operation, it is believed that the prolines introduce a localized area of rigidity and lend stability to the immunoglobulin molecule, particularly to the regions around the antigen combining sites.

[0052] Thus, the invention provides for a method to increase the affinity of antibodies comprising replacing amino acids of the variable domain heavy and/or light chain with proline or hydroxyproline (collectively referred to as "proline"). In some embodiments, the substitution of prolines is in the heavy chain variable domain. In some embodiments, the substitution of prolines is in the light chain variable domain. In other embodiments, the substitution of proline is in both the heavy chain and the light chain of the variable domain of the immunoglobulin molecule. In some embodiments, the proline substitutes for another amino acid having a non-polar sidechain (*e.g.*, glycine, alanine, valine, leucine, isoleucine, phenylalanine, methionine, tryptophan and cysteine). In some embodiments, further exchanges of amino acids having non-polar sidechains with other amino acids having non-polar sidechains may also confer increased affinity of the antibody for the antigen. In some embodiments, the amino acid substitutions are in a framework region of the heavy chain. In other embodiments, the amino acid substitutions are in a framework region of the light chain. In other embodiments, the amino acid substitutions are in a framework region of both the heavy and light chain. In some embodiments, the amino acid substitutions are in the first framework region (FR1) of the heavy chain. In other embodiments, the amino acid substitution is in the second framework region (FR2) of the heavy chain. In other embodiments, the amino acid substitution is in the third framework region (FR3) of the heavy chain. In other embodiments, the amino acid substitution is in the fourth framework region (FR4) of the heavy chain. In some embodiments, the amino acid substitutions are in the first framework region (FR1) of the light chain. In other embodiments, the amino acid substitution is in the second framework region (FR2) of the light chain. In other embodiments, the amino acid substitution is in the third framework region (FR3) of the light chain. In other embodiments, the amino acid substitution is in the fourth framework region (FR4) of the light chain.

[0053] In certain embodiments of the invention, a proline substitutes for an alanine at position 6 of SEQ ID NO:18. In other embodiments, proline substitutes for alanine at position 6 of SEQ ID NO:18 and the glycine at position 9 of SEQ ID NO:18, and/or the lysine at position 10 of SEQ ID NO:18 is substituted with an amino acid having a non-polar side chain (preferably, valine and arginine, respectively). In other embodiments, proline substitutes for leucine at position 22 of SEQ ID NO:21. For further information on the background of the invention the following references may be consulted, each of which is incorporated herein by reference in its entirety:

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16. Nicolaides, N.C. *et al.* (1998) A Naturally Occurring hPMS2 Mutation Can Confer a Dominant Negative Mutator Phenotype. *Mol. Cell. Biol.* 18:1635-1641.

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[0054] The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1: Stable expression of dominant negative MMR genes in hybridoma cells

[0055] It has been previously shown by Nicolaides et al. (Nicolaides *et al.* (1998) A Naturally Occurring hPMS2 Mutation Can Confer a Dominant Negative Mutator Phenotype *Mol. Cell. Biol.* 18:1635-1641) that the expression of a dominant negative allele in an otherwise MMR proficient cell could render these host cells MMR deficient. The creation of MMR deficient cells can lead to the generation of genetic alterations throughout the entire genome of a host organism's offspring, yielding a population of genetically altered offspring or siblings that may produce biochemicals with altered properties. This patent application teaches of the use of dominant negative MMR genes in antibody-producing cells, including but not limited to rodent hybridomas, human hybridomas, chimeric rodent cells producing human immunoglobulin gene products, human cells expressing immunoglobulin genes, mammalian cells producing single chain antibodies, and prokaryotic cells producing mammalian immunoglobulin genes or chimeric immunoglobulin molecules such as those contained within single-chain antibodies. The cell expression systems described above that are used to produce antibodies are well known by those skilled in the art of antibody therapeutics.

[0056] To demonstrate the ability to create MMR defective hybridomas using dominant negative alleles of MMR genes, we first transfected a mouse hybridoma cell line that is known to produce an antibody directed against the human IgE protein with an expression vector containing the human PMS2 (cell line referred to as HBPMS2), the previously published dominant negative PMS2 mutant referred herein as PMS134 (cell line referred to as HB134), or with no insert (cell line referred to as HBvec). The results showed that the PMS134 mutant could indeed exert a robust dominant negative effect, resulting in biochemical and genetic manifestations of MMR deficiency. Unexpected was the finding that the full length PMS2 also resulted in a lower MMR activity while no effect was seen in cells containing the empty vector. A brief description of the methods is provided below.

[0057] The MMR proficient mouse H36 hybridoma cell line was transfected with various *hPMS2* expression plasmids plus reporter constructs for assessing MMR activity. The MMR genes were cloned into the pEF expression vector, which contains the elongation factor promoter upstream of the cloning site followed by a mammalian polyadenylation signal. This

vector also contains the NEOr gene that allows for selection of cells retaining this plasmid. Briefly, cells were transfected with 1 μg of each vector using polyliposomes following the manufacturer's protocol (Life Technologies). Cells were then selected in 0.5 mg/ml of G418 for 10 days and G418 resistant cells were pooled together to analyze for gene expression. The pEF construct contains an intron that separates the exon 1 of the EF gene from exon 2, which is juxtaposed to the 5' end of the polylinker cloning site. This allows for a rapid reverse transcriptase polymerase chain reaction (RT-PCR) screen for cells expressing the spliced products. At day 17, 100,000 cells were isolated and their RNA extracted using the trizol method as previously described (Nicolaides N.C., Kinzler, K.W., and Vogelstein, B. (1995) Analysis of the 5' region of PMS2 reveals heterogeneous transcripts and a novel overlapping gene. *Genomics* 29:329-334). RNAs were reverse transcribed using Superscript II (Life Technologies) and PCR amplified using a sense primer located in exon 1 of the EF gene (5'-ttt cgc aac ggg ttt gcc g-3') (SEQ ID NO:23) and an antisense primer (5'-gtt tca gag tta agc ctt cg-3') (SEQ ID NO:24) centered at nt 283 of the published human PMS2 cDNA, which will detect both the full length as well as the PMS134 gene expression. Reactions were carried out using buffers and conditions as previously described (Nicolaides, N.C., *et al.* (1995) Genomic organization of the human PMS2 gene family. *Genomics* 30:195-206), using the following amplification parameters: 94°C for 30 sec, 52°C for 2 min, 72°C for 2 min, for 30 cycles. Reactions were analyzed on agarose gels. Figure 1 shows a representative example of PMS expression in stably transduced H36 cells.

[0058] Expression of the protein encoded by these genes were confirmed via western blot using a polyclonal antibody directed to the first 20 amino acids located in the N-terminus of the protein following the procedures previously described (data not shown) (Nicolaides *et al.* (1998) A Naturally Occurring hPMS2 Mutation Can Confer a Dominant Negative Mutator Phenotype. *Mol. Cell. Biol.* 18:1635-1641.

EXAMPLE 2: hPMS134 Causes a Defect in MMR Activity and hypermutability in hybridoma cells

[0059] A hallmark of MMR deficiency is the generation of unstable microsatellite repeats in the genome of host cells. This phenotype is referred to as microsatellite instability (MI)

(Modrich, P. (1994) Mismatch repair, genetic stability, and cancer *Science* 266:1959-1960; Palombo, F., *et al.* (1994) Mismatch repair and cancer *Nature* 36:417). MI consists of deletions and/or insertions within repetitive mono-, di- and/or tri nucleotide repetitive sequences throughout the entire genome of a host cell. Extensive genetic analyses of eukaryotic cells have found that the only biochemical defect that is capable of producing MI is defective MMR (Strand, M., *et al.* (1993) Destabilization of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair *Nature* 365:274-276; Perucho, M. (1996) Cancer of the microsatellite mutator phenotype. *Biol Chem.* 377:675-684; Eshleman J.R., and Markowitz, S.D. (1996) Mismatch repair defects in human carcinogenesis. *Hum. Mol. Genet.* 5:1489-494). In light of this unique feature that defective MMR has on promoting MI, it is now used as a biochemical marker to survey for lack of MMR activity within host cells (Perucho, M. (1996) Cancer of the microsatellite mutator phenotype. *Biol Chem.* 377:675-684; Eshleman J.R., and Markowitz, S.D. (1996) Mismatch repair defects in human carcinogenesis. *Hum. Mol. Genet.* 5:1489-494; Liu, T., *et al.* (2000) Microsatellite instability as a predictor of a mutation in a DNA mismatch repair gene in familial colorectal cancer *Genes Chromosomes Cancer* 27:17-25).

[0060] A method used to detect MMR deficiency in eukaryotic cells is to employ a reporter gene that has a polynucleotide repeat inserted within the coding region that disrupts its reading frame due to a frame shift. In the case where MMR is defective, the reporter gene will acquire random mutations (i.e. insertions and/or deletions) within the polynucleotide repeat yielding clones that contain a reporter with an open reading frame. We have employed the use of an MMR-sensitive reporter gene to measure for MMR activity in HBvec, HBPMS2, and HBPMS134 cells. The reporter construct used the pCAR-OF, which contains a hygromycin resistance (HYG) gene plus a β-galactosidase gene containing a 29 bp out-of-frame poly-CA tract at the 5' end of its coding region. The pCAR-OF reporter would not generate β -galactosidase activity unless a frame-restoring mutation (*i.e.*, insertion or deletion) arose following transfection. HBvec, HBPMS2, and HB134 cells were each transfected with pCAR-OF vector in duplicate reactions following the protocol described in Example 1. Cells were selected in 0.5 mg/ml G418 and 0.5mg/ml HYG to select for cells retaining both the MMR effector and the pCAR-OF reporter plasmids. All cultures

transfected with the pCAR vector resulted in a similar number of HYG/G418 resistant cells. Cultures were then expanded and tested for β-galactosidase activity *in situ* as well as by biochemical analysis of cell extracts. For *in situ* analysis, 100,000 cells were harvested and fixed in 1% gluteraldehyde, washed in phosphate buffered saline solution and incubated in 1 ml of X-gal substrate solution [0.15 M NaCl, 1 mM $MgCl_2$, 3.3 mM $K_4Fe(CN)_6$, 3.3 mM $K_3Fe(CN)_6$, 0.2% X-Gal] in 24 well plates for 2 hours at 37°C. Reactions were stopped in 500 mM sodium bicarbonate solution and transferred to microscope slides for analysis. Three fields of 200 cells each were counted for blue (β-galactosidase positive cells) or white (β-galactosidase negative cells) to assess for MMR inactivation. Table 1 shows the results from these studies. While no β-galactosidase positive cells were observed in HBvec cells, 10% of the cells per field were β-galactosidase positive in HB134 cultures and 2% of the cells per field were β-galactosidase positive in HBPMS2 cultures.

[0061] Cell extracts were prepared from the above cultures to measure β-galactosidase using a quantitative biochemical assay as previously described (Nicolaides *et al.* (1998) A Naturally Occurring hPMS2 Mutation Can Confer a Dominant Negative Mutator Phenotype *Mol. Cell. Biol.* 18:1635-1641; Nicolaides, N.C., *et al.* (1992) The Jun family members, c-JUN and JUND, transactivate the human *c-myb* promoter via an Ap1 like element. *J. Biol. Chem.* 267:19665-19672). Briefly, 100,000 cells were collected, centrifuged and resuspended in 200 µls of 0.25M Tris, pH 8.0. Cells were lysed by freeze/thawing three times and supernatants collected after microfugation at 14,000 rpms to remove cell debris. Protein content was determined by spectrophotometric analysis at OD^{280}. For biochemical assays, 20 µg of protein was added to buffer containing 45 mM 2-mercaptoethanol, 1mM $MgCl_2$, 0.1 M $NaPO_4$ and 0.6 mg/ml Chlorophenol red-β-D-galactopyranoside (CPRG, Boehringer Mannheim). Reactions were incubated for 1 hour, terminated by the addition of 0.5 M Na_2CO_3, and analyzed by spectrophotometry at 576 nm. H36 cell lysates were used to subtract out background. Figure 2 shows the β-galactosidase activity in extracts from the various cell lines. As shown, the HB134 cells produced the highest amount of β-galactosidase, while no activity was found in the HBvec cells containing the pCAR-OF.

These data demonstrate the ability to generate MMR defective hybridoma cells using dominant negative MMR gene alleles.

Table 1. β-galactosidase expression of HBvec, HBPMS2 and HB134 cells transfected with pCAR-OF reporter vectors. Cells were transfected with the pCAR-OF β-galactosidase reporter plasmid. Transfected cells were selected in hygromycin and G418, expanded and stained with X-gal solution to measure for β-galactosidase activity (blue colored cells). 3 fields of 200 cells each were analyzed by microscopy. The results below represent the mean +/- standard deviation of these experiments.

Table 1.

CELL LINE	# BLUE CELLS
HBvec	0 +/- 0
HBPMS2	4 +/- 1
HB134	20 +/- 3

EXAMPLE 3: Screening strategy to identify hybridoma clones producing antibodies with higher binding affinities and/or increased immunoglobulin production.

[0062] An application of the methods presented within this document is the use of MMR deficient hybridomas or other immunoglobulin producing cells to create genetic alterations within an immunoglobulin gene that will yield antibodies with altered biochemical properties. An illustration of this application is demonstrated within this example whereby the HB134 hybridoma (see Example 1), which is a MMR-defective cell line that produces an anti-human immunoglobulin type E (hIgE) MAb, is grown for 20 generations and clones are isolated in 96-well plates and screened for hIgE binding. Figure 3 outlines the screening procedure to identify clones that produce high affinity MAbs, which is presumed to be due to an alteration within the light or heavy chain variable region of the protein. The assay employs the use of a plate Enzyme Linked Immunosorbant Assay (ELISA) to screen for clones that produce high-affinity MAbs. 96-well plates containing single cells from HBvec or HB134 pools are grown

for 9 days in growth medium (RPMI 1640 plus 10% fetal bovine serum) plus 0.5 mg/ml G418 to ensure clones retain the expression vector. After 9 days, plates are screened using a hIgE plate ELISA, whereby a 96 well plate is coated with 50µls of a 1 µg/ml hIgE solution for 4 hours at 4°C. Plates are washed 3 times in calcium and magnesium free phosphate buffered saline solution ($PBS^{-/-}$) and blocked in 100µls of $PBS^{-/-}$ with 5% dry milk for 1 hour at room temperature. Wells are rinsed and incubated with 100 µls of a PBS solution containing a 1:5 dilution of conditioned medium from each cell clone for 2 hours. Plates are then washed 3 times with $PBS^{-/-}$ and incubated for 1 hour at room temperature with 50 µls of a $PBS^{-/-}$ solution containing 1:3000 dilution of a sheep anti-mouse horse radish peroxidase (HRP) conjugated secondary antibody. Plates are then washed 3 times with $PBS^{-/-}$ and incubated with 50 µls of TMB-HRP substrate (BioRad) for 15 minutes at room temperature to detect amount of antibody produced by each clone. Reactions are stopped by adding 50 µls of 500mM sodium bicarbonate and analyzed by OD at 415nm using a BioRad plate reader. Clones exhibiting an enhanced signal over background cells (H36 control cells) are then isolated and expanded into 10 ml cultures for additional characterization and confirmation of ELISA data in triplicate experiments. ELISAs are also performed on conditioned medium (CM) from the same clones to measure total Ig production within the conditioned medium of each well. Clones that produce an increased ELISA signal and have increased antibody levels are then further analyzed for variants that over-express and/or over-secrete antibodies as described in Example 4. Analysis of five 96-well plates each from HBvec or HB134 cells have found that a significant number of clones with a higher Optical Density (OD) value is observed in the MMR-defective HB134 cells as compared to the HBvec controls. Figure 4 shows a representative example of HB134 clones producing antibodies that bind to specific antigen (in this case IgE) with a higher affinity. Figure 4 provides raw data from the analysis of 96 wells of HBvec (left graph) or HB134 (right graph) which shows 2 clones from the HB134 plate to have a higher OD reading due to 1) genetic alteration of the antibody variable domain that leads to an increased binding to IgE antigen, or 2) genetic alteration of a cell host that leads to over-production/secretion of the antibody molecule. Anti-Ig ELISA found that the two clones, shown in Figure 4 have Ig levels within their CM similar to the surrounding wells exhibiting ower OD values. These data suggest

that a genetic alteration occurred within the antigen binding domain of the antibody which in turn allows for higher binding to antigen.

[0063] Clones that produced higher OD values as determined by ELISA were further analyzed at the genetic level to confirm that mutations within the light or heavy chain variable region have occurred that lead to a higher binding affinity hence yielding to a stronger ELISA signal. Briefly, 100,000 cells are harvested and extracted for RNA using the Triazol method as described above. RNAs are reverse transcribed using Superscript II as suggested by the manufacturer (Life Technology) and PCR amplified for the antigen binding sites contained within the variable light and heavy chains. Because of the heterogeneous nature of these genes, the following degenerate primers are used to amplify light and heavy chain alleles from the parent H36 strain.

Light chain sense: 5'-GGA TTT TCA GGT GCA GAT TTT CAG-3' (SEQ ID NO:1)

Light chain antisense: 5'-ACT GGA TGG TGG GAA GAT GGA-3' (SEQ ID NO:2)

Heavy chain sense: 5'-A(G/T) GTN (A/C)AG CTN CAG (C/G)AG TC-3' (SEQ ID NO:3)

Heavy chain antisense: 5'-TNC CTT G(A/G)C CCC AGT A(G/A)(A/T)C-3' (SEQ ID NO:4)

[0064] PCR reactions using degenerate oligonucleotides are carried out at 94°C for 30 sec, 52°C for 1 min, and 72°C for 1 min for 35 cycles. Products are analyzed on agarose gels. Products of the expected molecular weights are purified from the gels by Gene Clean (Bio 101), cloned into T-tailed vectors, and sequenced to identify the wild type sequence of the variable light and heavy chains. Once the wild type sequence has been determined, non-degenerate primers were made for RT-PCR amplification of positive HB134 clones. Both the light and heavy chains were amplified, gel purified and sequenced using the corresponding sense and antisense primers. The sequencing of RT-PCR products gives representative sequence data of the endogenous immunoglobulin gene and not due to PCR induced mutations. Sequences from clones were then compared to the wild type sequence for sequence comparison. An example of the ability to create *in vivo* mutations within an

immunoglobulin light or heavy chain is shown in Figure 5, where HB134 clone92 was identified by ELISA to have an increased signal for hIgE. The light chain was amplified using specific sense and antisense primers. The light chain was RT-PCR amplified and the resulting product was purified and analyzed on an automated ABI377 sequencer. As shown in clone A, a residue –4 upstream of the CDR region 3 had a genetic change from ACT to TCT, which results in a Thr to Ser change within the framework region just preceding the CDR#3. In clone B, a residue –6 upstream of the CDR region had a genetic change from CCC to CTC, which results in a Pro to His change within framework region preceding CDR#2.

[0065] The ability to generate random mutations in immunoglobulin genes or chimeric immunoglobulin genes is not limited to hybridomas. Nicolaides et al. (Nicolaides *et al.* (1998) A Naturally Occurring hPMS2 Mutation Can Confer a Dominant Negative Mutator Phenotype *Mol. Cell. Biol.* 18:1635-1641) has previously shown the ability to generate hypermutable hamster cells and produce mutations within an endogenous gene. A common method for producing humanized antibodies is to graft CDR sequences from a MAb (produced by immunizing a rodent host) onto a human Ig backbone, and transfection of the chimeric genes into Chinese Hamster Ovary (CHO) cells whih in turn produce a functional Ab that is secreted by the CHO cells (Shields, R.L., *et al.* (1995) Anti-IgE monoclonal antibodies that inhibit allergen-specific histamine release. *Int. Arch. Allergy Immunol.* 107:412-413). The methods described within this application are also useful for generating genetic alterations within Ig genes or chimeric Igs transfected within host cells such as rodent cell lines, plants, yeast and prokaryotes (Frigerio L, *et al.* (2000) Assembly, secretion, and vacuolar delivery of a hybrid immunoglobulin in plants. *Plant Physiol.* 123:1483-1494).

[0066] These data demonstrate the ability to generate hypermutable hybridomas, or other Ig producing host cells that can be grown and selected, to identify structurally altered immunoglobulins yielding antibodies with enhanced biochemical properties, including but not limited to increased antigen binding affinity. Moreover, hypermutable clones that contain missense mutations within the immunoglobulin gene that result in an amino acid change or changes can be then further characterized for *in vivo* stability, antigen clearance, on-off

binding to antigens, etc. Clones can also be further expanded for subsequent rounds of *in vivo* mutations and can be screened using the strategy listed above.

[0067] The use of chemical mutagens to produce genetic mutations in cells or whole organisms are limited due to the toxic effects that these agents have on "normal" cells. The use of chemical mutagens such as MNU in MMR defective organisms is much more tolerable yielding to a 10 to 100 fold increase in genetic mutation over MMR deficiency alone (Bignami M, (2000) Unmasking a killer: DNA O(6)-methylguanine and the cytotoxicity of methylating agents. *Mutat. Res.* 462:71-82). This strategy allows for the use of chemical mutagens to be used in MMR-defective Ab producing cells as a method for increasing additional mutations within immunoglobulin genes or chimeras that may yield functional Abs with altered biochemical properties such as enhanced binding affinity to antigen, etc.

Example 4: Generation of antibody producing cells with enhanced antibody production

[0068] Analysis of clones from H36 and HB134 following the screening strategy listed above hasidentified a significant number of clones that produce enhanced amounts of antibody into the medium. While a subset of these clones gave higher Ig binding data as determined by ELISA as a consequence of mutations within the antigen binding domains contained in the variable regions, others were found to contain "enhanced" antibody production. A summary of the clones producing enhanced amounts of secreted MAb is shown in TABLE 2, where a significant number of clones from HB134 cells were found to produce enhanced Ab production within the conditioned medium as compared to H36 control cells.

TABLE 2. Generation of hybridoma cells producing high levels of antibody. HB134 clones were assayed by ELISA for elevated Ig levels. Analysis of 480 clones showed that a significant number of clones had elevated MAb product levels in their CM. Quantification showed that several of these clones produced greater than 500ngs/ml of MAb due to either enhanced expression and/or secretion as compared to clones from the H36 cell line.

Table 2. Production of MAb in CM from H36 and HB134 clones.

Cell Line	% clones > 400 ng/ml	% clones >500 ng/ml
H36	1/480 = 0.2%	0/480 = 0%

HB134	50/480 = 10%	8/480 = 1.7%

[0069] Cellular analysis of HB134 clones with higher MAb levels within the conditioned medium (CM) were analyzed to determine if the increased production was simply due to genetic alterations at the Ig locus that may lead to over-expression of the polypeptides forming the antibody, or due to enhanced secretion due to a genetic alteration affecting secretory pathway mechanisms. To address this issue, we expanded three HB134 clones that had increased levels of antibody within their CM. 10,000 cells were prepared for western blot analysis to assay for intracellular steady state Ig protein levels (Figure 6). In addition, H36 cells were used as a standard reference (Lane 2) and a rodent fibroblast (Lane 1) was used as an Ig negative control. Briefly, cells were pelleted by centrifugation and lysed directly in 300 ul of SDS lysis buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1 M 2-mercaptoethanol, 0.001% bromophenol blue) and boiled for 5 minutes. Lysate proteins were separated by electrophoresis on 4-12% NuPAGE gels (for analysis of Ig heavy chain. Gels were electroblotted onto Immobilon-P (Millipore) in 48 mM Tris base, 40 mM glycine, 0.0375% SDS, 20% methanol and blocked at room temperature for 1 hour in Tris-buffered saline (TBS) plus 0.05% Tween-20 and 5% condensed milk. Filters were probed with a 1:10,000 dilution of sheep anti-mouse horseradish peroxidase conjugated monoclonal antibody in TBS buffer and detected by chemiluminescence using Supersignal substrate (Pierce). Experiments were repeated in duplicates to ensure reproducibility. Figure 6 shows a representative analysis where a subset of clones had enhanced Ig production which accounted for increased Ab production (Lane 5) while others had a similar steady state level as the control sample, yet had higher levels of Ab within the CM. These data suggest a mechanism whereby a subset of HB134 clones contained a genetic alteration that in turn produces elevated secretion of antibody.

[0070] The use of chemical mutagens to produce genetic mutations in cells or whole organisms are limited due to the toxic effects that these agents have on "normal" cells. The use of chemical mutagens such as MNU in MMR defective organisms is much more tolerable yielding to a 10 to 100 fold increase in genetic mutation over MMR deficiency alone (Bignami M, (2000) Unmasking a killer: DNA O(6)-methylguanine and the cytotoxicity of

methylating agents. *Mutat. Res.* 462:71-82). This strategy allows for the use of chemical mutagens to be used in MMR-defective Ab producing cells as a method for increasing additional mutations within immunoglobulin genes or chimeras that may yield functional Abs with altered biochemical properties such as enhanced binding affinity to antigen, etc.

Example 5: Establishment of genetic stability in hybridoma cells with new output trait.

[0071] The initial steps of MMR are dependent on two protein complexes, called MutSα and MutLα (Nicolaides *et al.* (1998) A Naturally Occurring hPMS2 Mutation Can Confer a Dominant Negative Mutator Phenotype. *Mol. Cell. Biol.* 18:1635-1641). Dominant negative MMR alleles are able to perturb the formation of these complexes with downstream biochemicals involved in the excision and polymerization of nucleotides comprising the "corrected" nucleotides. Examples from this application show the ability of a truncated MMR allele (PMS134) as well as a full length human PMS2 when expressed in a hybridoma cell line is capable of blocking MMR resulting in a hypermutable cell line that gains genetic alterations throughout its entire genome per cell division. Once a cell line is produced that contains genetic alterations within genes encoding for an antibody, a single chain antibody, over expression of immunoglobulin genes and/or enhanced secretion of antibody, it is desirable to restore the genomic integrity of the cell host. This can be achieved by the use of inducible vectors whereby dominant negative MMR genes are cloned into such vectors, introduced into Ab producing cells and the cells are cultured in the presence of inducer molecules and/or conditions. Inducible vectors include but are not limited to chemical regulated promoters such as the steroid inducible MMTV, tetracycline regulated promoters, temperature sensitive MMR gene alleles, and temperature sensitive promoters.

[0072] The results described above lead to several conclusions. First, expression of hPMS2 and PMS134 results in an increase in microsatellite instability in hybridoma cells. That this elevated microsatellite instability is due to MMR deficiency was proven by evaluation of extracts from stably transduced cells. The expression of PMS134 results in a polar defect in MMR, which was only observed using heteroduplexes designed to test repair from the 5' direction (no significant defect in repair from the 3' direction was observed in the same extracts) (Nicolaides *et al.* (1998) A Naturally Occurring hPMS2 Mutation Can Confer a

Dominant Negative Mutator Phenotype. *Mol. Cell. Biol.* 18:1635-1641). Interestingly, cells deficient in hMLH1 also have a polar defect in MMR, but in this case preferentially affecting repair from the 3' direction (Drummond, J.T, *et al.* (1996) Cisplatin and adriamycin resistance are associated with MutLa and mismatch repair deficiency in an ovarian tumor cell line. *J. Biol. Chem.* 271:9645-19648). It is known from previous studies in both prokaryotes and eukaryotes that the separate enzymatic components mediate repair from the two different directions. Our results, in combination with those of Drummond *et al.*(Shields, R.L., *et al.* (1995) Anti-IgE monoclonal antibodies that inhibit allergen-specific histamine release. *Int. Arch Allergy Immunol.* 107:412-413), strongly suggest a model in which 5' repair is primarily dependent on hPMS2 while 3' repair is primarily dependent on hMLH1. It is easy to envision how the dimeric complex between PMS2 and MLH1 might set up this directionality. The combined results also demonstrate that a defect in directional MMR is sufficient to produce a MMR defective phenotype and suggests that any MMR gene allele is useful to produce genetically altered hybridoma cells, or a cell line that is producing Ig gene products. Moreover, the use of such MMR alleles will be useful for generating genetically altered Ig polypeptides with altered biochemical properties as well as cell hosts that produce enhanced amounts of antibody molecules.

[0073] Another method that is taught in this application is that ANY method used to block MMR can be performed to generate hypermutablility in an antibody-producing cell that can lead to genetically altered antibodies with enhanced biochemical features such as but not limited to increased antigen binding, enhanced pharmacokinetic profiles, etc. These processes can also to be used to generate antibody producer cells that have increased Ig expression as shown in Example 4, Figure 6 and/or increased antibody secretion as shown in Table 2.

[0074] In addition, we demonstrate the utility of blocking MMR in antibody producing cells to increase genetic alterations within Ig genes that may lead to altered biochemical features such as, but not limited to, increased antigen binding affinities (Figure 5A and 5B). The blockade of MMR in such cells can be through the use of dominant negative MMR gene alleles from any species including bacteria, yeast, protozoa, insects, rodents, primates, mammalian cells, and man. Blockade of MMR can also be generated through the use of

antisense RNA or deoxynucleotides directed to any of the genes involved in the MMR biochemical pathway. Blockade of MMR can be through the use of polypeptides that interfere with subunits of the MMR complex including but not limited to antibodies. Finally, the blockade of MMR may be through the use chemicals such as but not limited to nonhydrolyzable ATP analogs, which have been shown to block MMR (Galio, L, *et al.* (1999) ATP hydrolysis-dependent formation of a dynamic ternary nucleoprotein complex with MutS and MutL. *Nucl. Acids Res.* 27:2325-23231).

Example 6: Analysis of Genetic Sequence of mutant H36 cell lines producing high affinity antibodies

[0075] The nucleic acid sequence of the light and heavy chains of the antibodies produced by the H36 mutant cell lines were examined for mutations within the immunoglobulin coding sequence that contribute to the increased affinity of the antibodies as compared to the parent clone. The results are shown in Table 3. The data show that proline substitutions in both the heavy and light chain variable domains contribute to increased affinity of the antibodies to antigen. A particular hot spot appears to be amino acid position 6 of SEQ ID NO:6 in which an amino acid substitution occurred changing the parental alanine to proline for HB91-47, HB134DRMA13, and HB134DRMA55. These three clones also had mutations at positions 9 and 10. In position 9, the parental valine was changed to glycine or arginine, while at position 10 of SEQ ID NO:6, the parental arginine was changed to lysine in both cases.

Table 3

Clones	Chain	Sequence Change	Amino Acid Change	Mean ELISA	Affinity
H36		WT	None	0.542	4.80E-08
HB-134al	L	A>T	Thr>Ser	1.632	Nd
HB91-34	H	C insertion	Frame-shift	0	0
HB91-37	L	T>C	Leu>Pro	1.743	1.40E-09
HB91-38	H	T>A	Ser>Ser	1.641	Nd
HB91-40	H	A>G	Ala>Thr	1.333	Nd
HB91-47	H	Multiple	Ala>Pro, Val>Gly, Arg>Lys	1.979	3.12E-09
HB91-53	H	TT>AA	Phe>Lys	1.144	Nd

HB91-62	H	A>G	Met>Gly	0.218	6.60E-07
HB91-71	H	T>G	Met>Gly	0.186	Nd
HB134DRMA13	H	Multiple	Ala>Pro, Val>Gly, Arg>Lys, Thr>Ala,	2.041	Nd
HB134DRMA14	H	G>A, A>G	Arg>Lys, Thr>Ala	1.211	Nd
HB134DRMA55	H	Multiple	Ala>Pro, Val>Arg, Arg>Lys, Thr>Glu, Ser>Thr	2.012	Nd

[0076] The genetically altered antibodies show the following sequence differences and consensus sequence:

Amino acid alignment of morphogenic HB91-47 heavy chain (SEQ ID NO:17), parental H36 heavy chain (SEQ ID NO:18), and consensus heavy chain sequence (SEQ ID NO:19)

```
                  1                                   35
Morphogenic  (1)  LQQSGPELGKPGTSVKISCKASGYTFTNYGMNWVK
H36 parental (1)  LQQSGAELVRPGTSVKISCKASGYTFTNYGMNWVK
Consensus    (1)  LQQSG EL  PGTSVKISCKASGYTFTNYGMNWVK
                  |          FR1              |CDR1 |

                  36                                  70
Morphogenic  (36) QAPGKGLKWMGWINTYTGEPTYADDFKGRFAFSLE
H36 parental (36) QAPGKGLKWMGWINTYTGEPTYADDFKGRFAFSLE
Consensus    (36) QAPGKGLKWMGWINTYTGEPTYADDFKGRFAFSLE
                      FR2     |       CDR2       | FR3
```

Amino acid alignment of morphogenic HB91-37 light chain (SEQ ID NO:20), parental H36 light chain (SEQ ID NO:21), and consensus light chain sequence (SEQ ID NO:22)

```
                  1                                   35
Morphogenic  (1)  SASSSVSSSYFHWYQQKSGASPKPLIHRTSNLASG
H36 parental (1)  SASSSVSSSYFHWYQQKSGASLKPLIHRTSNLASG
Consensus    (1)  SASSSVSSSYFHWYQQKSGAS KPLIHRTSNLASG
                     CDR1     |       FR2       | CDR2 |

                  36        45
Morphogenic  (36) VPARFSGSGS
H36 parental (36) VPARFSGSGS
Consensus    (36) VPARFSGSGS
```

FR3

[0077] The data shows that for the light chain, a substitution in the second framework region (FR2) of the light chain at position 22 of SEQ ID NO:21 to a proline increased the binding affinity of the antibody.

WE CLAIM:

1. A method of increasing affinity of a monoclonal antibody for an antigen comprising substituting an amino acid within the variable domain of the heavy or light chain of said monoclonal antibody with a second amino acid having a non-polar side chain, whereby said affinity of said monoclonal antibody for said antigen is increased.
2. The method of claim 1 wherein said second amino acid is a proline.
3. The method of claim 2 wherein said amino acid within the variable domain of the heavy or light chain of said monoclonal antibody is an amino acid having a non-polar side chain.
4. The method of claim 3 wherein said amino acid is an alanine, or leucine.
5. The method of claim 3 wherein said amino acid is in the first framework region of the heavy chain of said monoclonal antibody.
6. The method of claim 3 wherein said amino acid is in the second framework region of the light chain of said monoclonal antibody.
7. The method of claim 2 wherein said amino acid is in position 6 of the first framework region as shown in SEQ ID NO:18.
8. The method of claim 2 wherein said amino acid is in position 22 of the second framework region of the light chain variable domain as shown in SEQ ID NO:21.
9. A method of increasing affinity of a monoclonal antibody for an antigen comprising substituting an amino acid within the variable domain of the heavy or light chain of said monoclonal antibody wherein said amino acid comprises a non-polar side chain, with a proline, whereby said affinity of said monoclonal antibody for said antigen is increased.
10. The method of claim 9 wherein said amino acid is an alanine, or leucine.
11. The method of claim 9 wherein said amino acid is in the first framework region of the heavy chain of said monoclonal antibody.
12. The method of claim 9 wherein said amino acid is in the second framework region of the light chain of said monoclonal antibody.
13. The method of claim 10 wherein said amino acid is in position 6 of the first framework region as shown in SEQ ID NO:18.

14. The method of claim 10 wherein said amino acid is in position 22 of the second framework region of the light chain variable domain as shown in SEQ ID NO:21.

15. A monoclonal antibody produced by the method of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14.

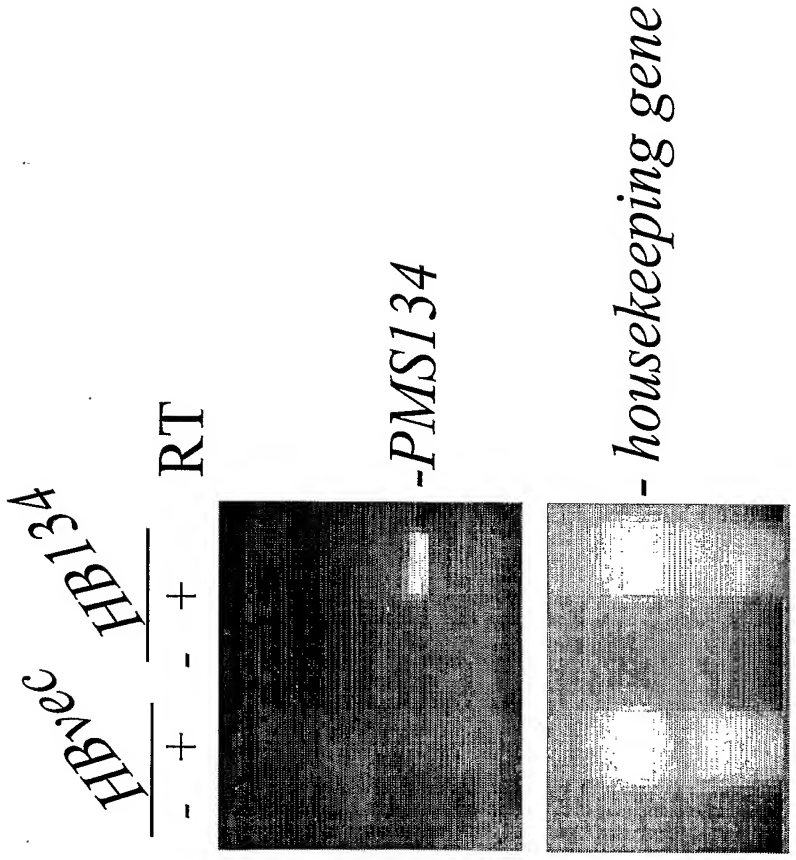
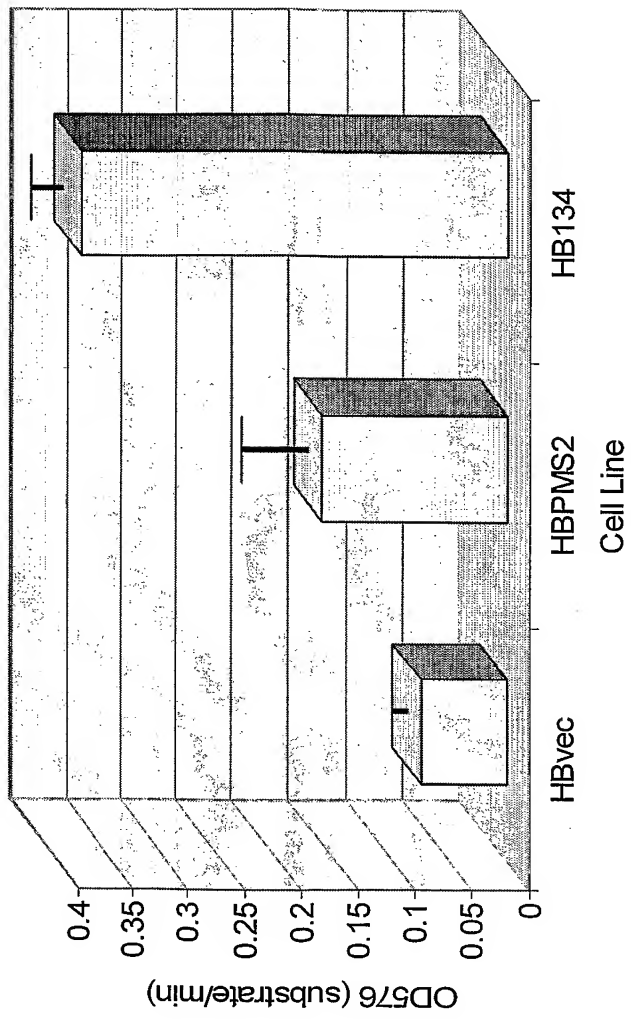


Fig. 1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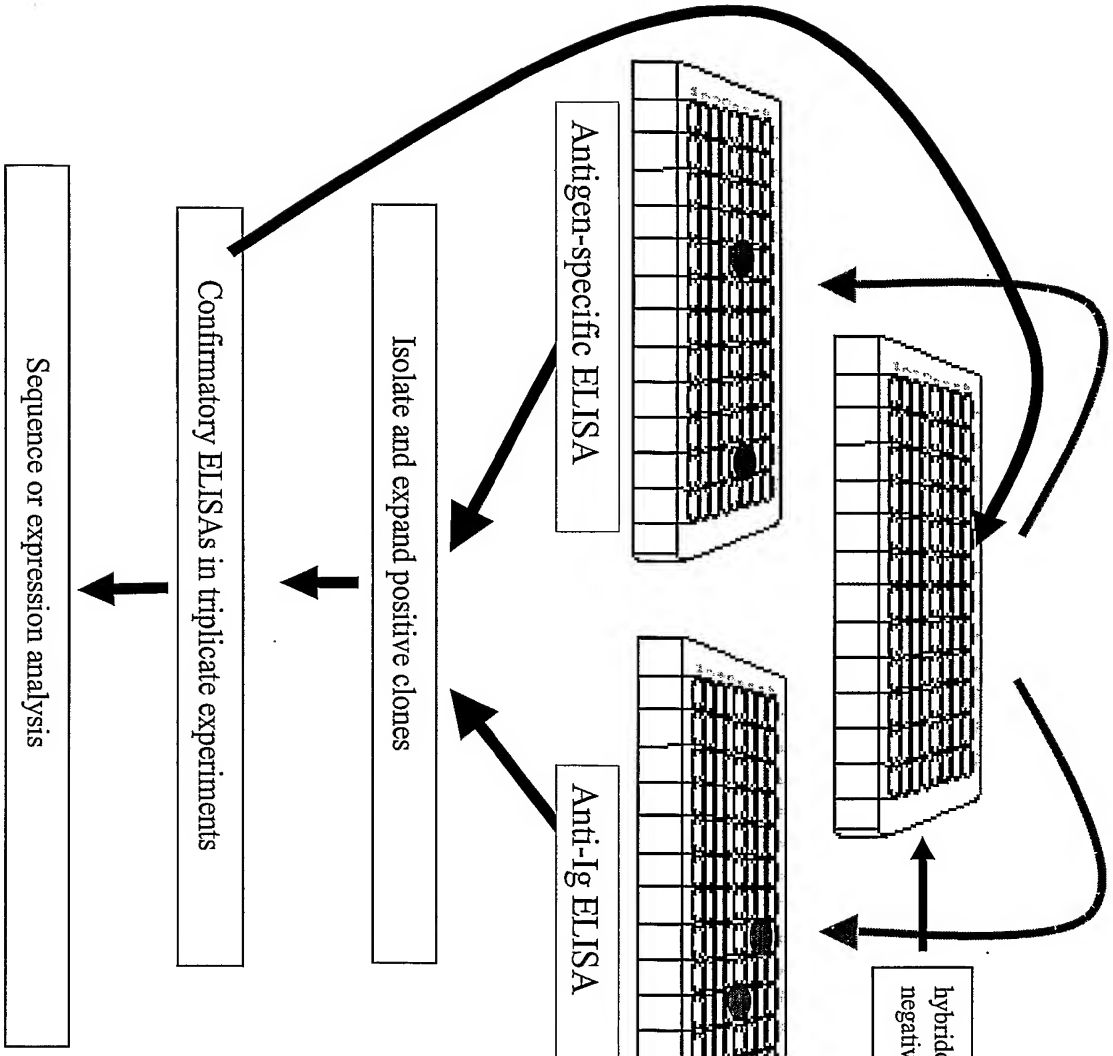
HBvec
HBPMS2
HB134
Cell Line

Fig. 2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Anti-Ig ELISA
Antigen-specific ELISA
Isolate and expand positive clones
Confirmatory ELISAs in triplicate experiments
Sequence or expression analysis

Fig. 3

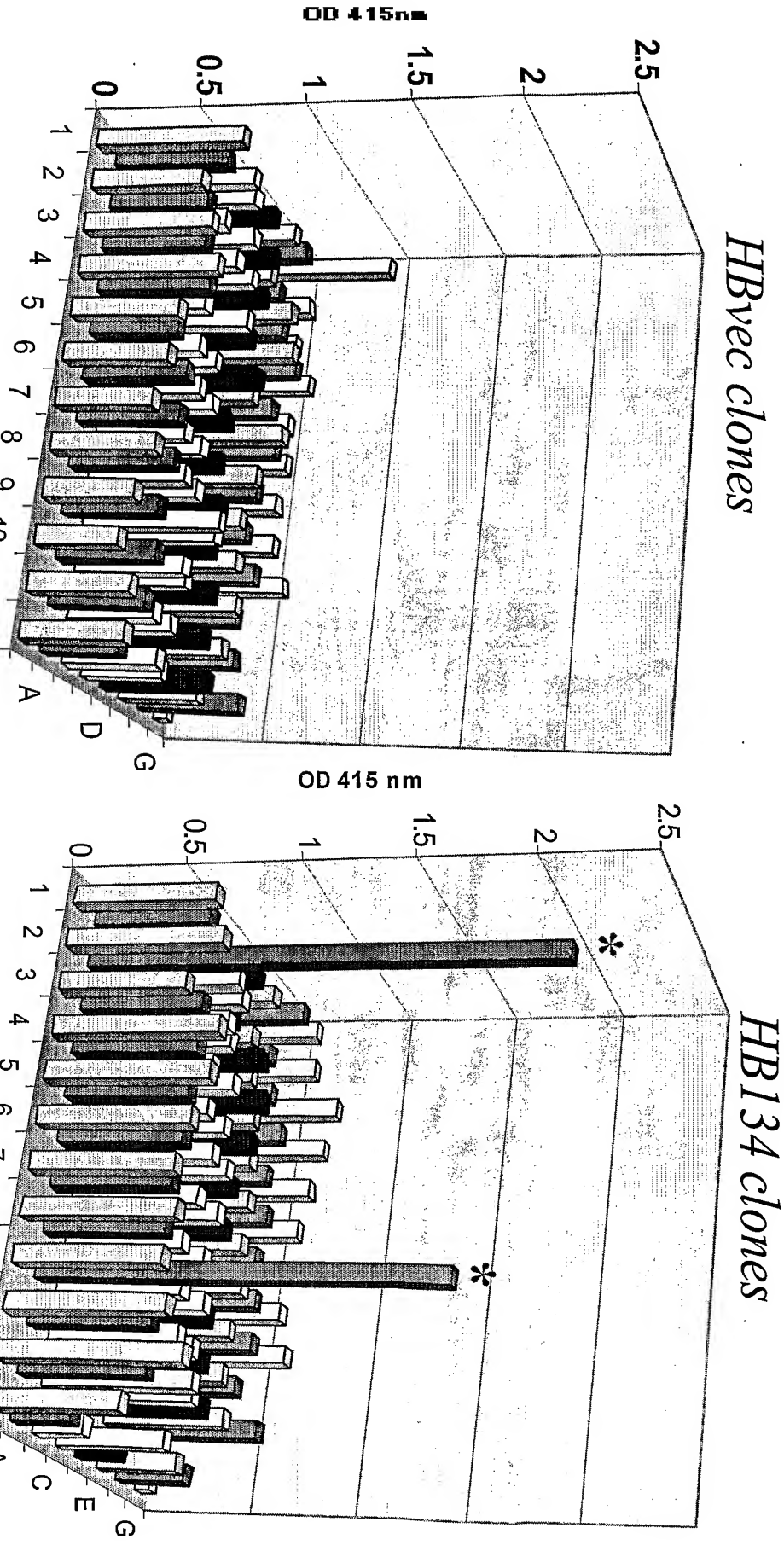
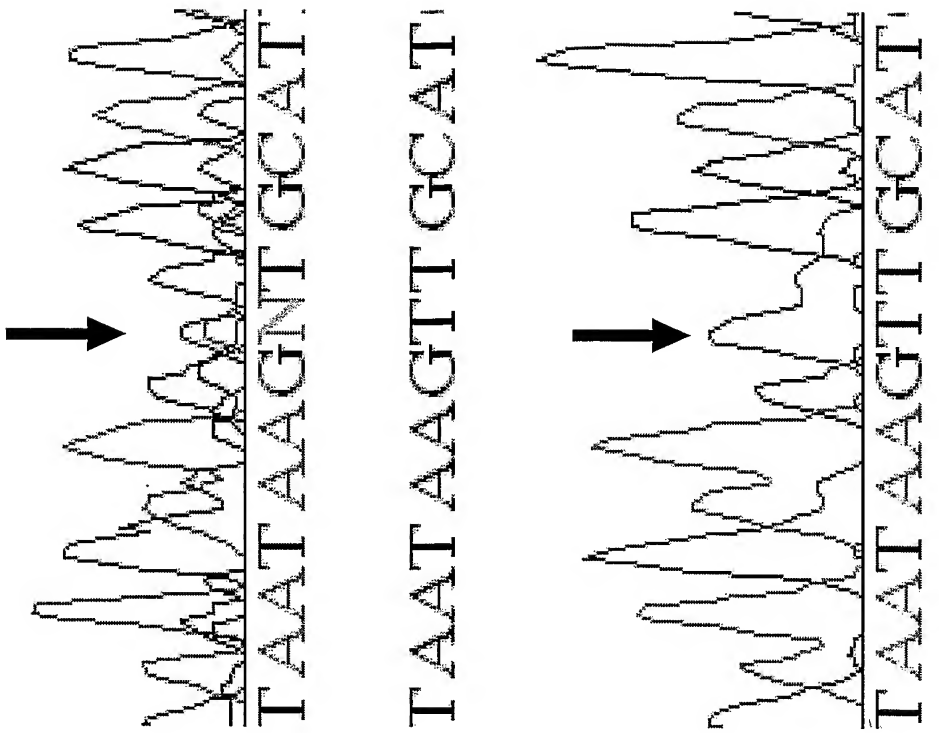


Fig. 4

* = clones with a significant difference in antigen binding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Consensus sequence
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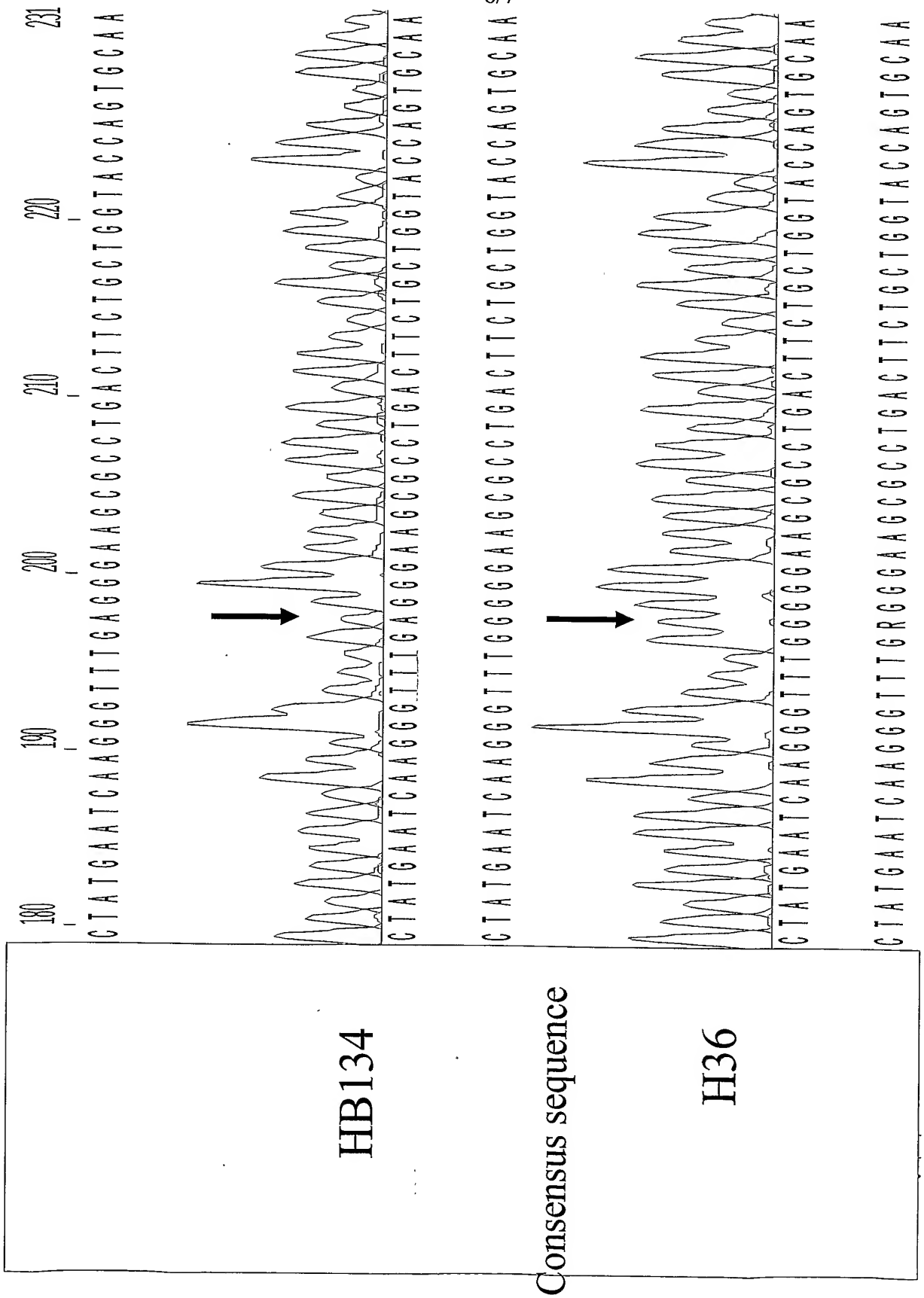
Fig. 5A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HB134
Consensus sequence
H36

Fig. 5B

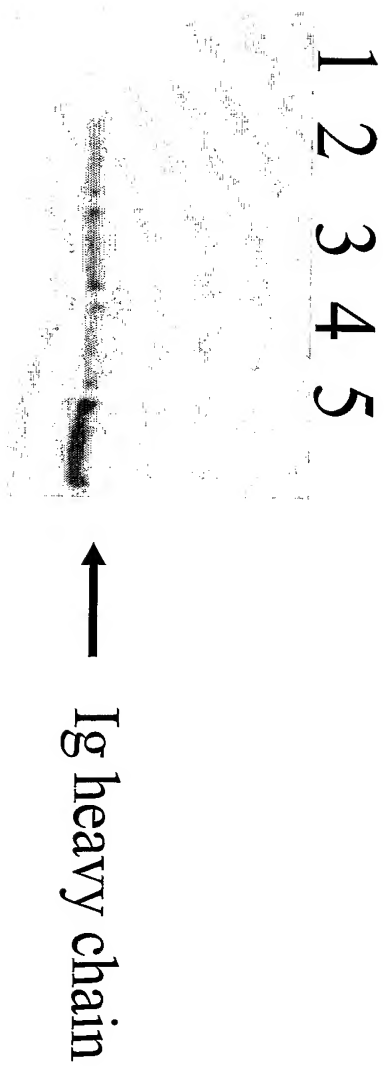

Fig. 6

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35 40 45

Ala Gly Ala Thr Asn Ile Asp Leu Lys Leu Lys Asp Tyr Gly Val Asp
50 55 60

Leu Ile Glu Val Ser Asp Asn Gly Cys Gly Val Glu Glu Glu Asn Phe
65 70 75 80

Glu Gly Leu Thr Leu Lys His His Thr Ser Lys Ile Gln Glu Phe Ala
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Asp Leu Thr Gln Val Glu Thr Phe Gly Phe Arg Gly Glu Ala Leu Ser
100 105 110

Ser Leu Cys Ala Leu Ser Asp Val Thr Ile Ser Thr Cys His Ala Ser
115 120 125

Ala Lys Val Gly Thr Arg Leu Met Phe Asp His Asn Gly Lys Ile Ile
130 135 140

Gln Lys Thr Pro Tyr Pro Arg Pro Arg Gly Thr Thr Val Ser Val Gln
145 150 155 160

Gln Leu Phe Ser Thr Leu Pro Val Arg His Lys Glu Phe Gln Arg Asn
165 170 175

Ile Lys Lys Glu Tyr Ala Lys Met Val Gln Val Leu His Ala Tyr Cys
180 185 190

Ile Ile Ser Ala Gly Ile Arg Val Ser Cys Thr Asn Gln Leu Gly Gln
195 200 205

Gly Lys Arg Gln Pro Val Val Cys Thr Gly Gly Ser Pro Ser Ile Lys
210 215 220

Glu Asn Ile Gly Ser Val Phe Gly Gln Lys Gln Leu Gln Ser Leu Ile
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Pro Phe Val Gln Leu Pro Pro Ser Asp Ser Val Cys Glu Glu Tyr Gly

245 250 255

Leu Ser Cys Ser Asp Ala Leu His Asn Leu Phe Tyr Ile Ser Gly Phe
260 265 270

Ile Ser Gln Cys Thr His Gly Val Gly Arg Ser Ser Thr Asp Arg Gln
275 280 285

Phe Phe Phe Ile Asn Arg Arg Pro Cys Asp Pro Ala Lys Val Cys Arg
290 295 300

Leu Val Asn Glu Val Tyr His Met Tyr Asn Arg His Gln Tyr Pro Phe
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Val Val Leu Asn Ile Ser Val Asp Ser Glu Cys Val Asp Ile Asn Val
325 330 335

Thr Pro Asp Lys Arg Gln Ile Leu Leu Gln Glu Glu Lys Leu Leu Leu
340 345 350

Ala Val Leu Lys Thr Ser Leu Ile Gly Met Phe Asp Ser Asp Val Asn
355 360 365

Lys Leu Asn Val Ser Gln Gln Pro Leu Leu Asp Val Glu Gly Asn Leu
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Ile Lys Met His Ala Ala Asp Leu Glu Lys Pro Met Val Glu Lys Gln
385 390 395 400

Asp Gln Ser Pro Ser Leu Arg Thr Gly Glu Glu Lys Lys Asp Val Ser
405 410 415

Ile Ser Arg Leu Arg Glu Ala Phe Ser Leu Arg His Thr Thr Glu Asn
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Lys Pro His Ser Pro Lys Thr Pro Glu Pro Arg Arg Ser Pro Leu Gly
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Gln Lys Arg Gly Met Leu Ser Ser Ser Thr Ser Gly Ala Ile Ser Asp
450 455 460

Lys Gly Val Leu Arg Pro Gln Lys Glu Ala Val Ser Ser Ser His Gly
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Gly Ser Thr Ser Val Asp Ser Glu Gly Phe Ser Ile Pro Asp Thr Gly
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Ser His Cys Ser Ser Glu Tyr Ala Ala Ser Ser Pro Gly Asp Arg Gly
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Lys Arg Phe Lys Lys Glu Glu Ile Leu Ser Ser Ser Asp Ile Cys Gln
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Lys Leu Val Asn Thr Gln Asp Met Ser Ala Ser Gln Val Asp Val Ala
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Val Lys Ile Asn Lys Lys Val Val Pro Leu Asp Phe Ser Met Ser Ser
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Leu Ala Lys Arg Ile Lys Gln Leu His His Glu Ala Gln Gln Ser Glu
625                 630                 635                 640

Gly Glu Gln Asn Tyr Arg Lys Phe Arg Ala Lys Ile Cys Pro Gly Glu
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Asn Gln Ala Ala Glu Asp Glu Leu Arg Lys Glu Ile Ser Lys Thr Met
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Phe Ala Glu Met Glu Ile Ile Gly Gln Phe Asn Leu Gly Phe Ile Ile
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Thr Lys Leu Asn Glu Asp Ile Phe Ile Val Asp Gln His Ala Thr Asp
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Glu Lys Tyr Asn Phe Glu Met Leu Gln Gln His Thr Val Leu Gln Gly
705                 710                 715                 720

Gln Arg Leu Ile Ala Pro Gln Thr Leu Asn Leu Thr Ala Val Asn Glu
                725                 730                 735

Ala Val Leu Ile Glu Asn Leu Glu Ile Phe Arg Lys Asn Gly Phe Asp
            740                 745                 750

Phe Val Ile Asp Glu Asn Ala Pro Val Thr Glu Arg Ala Lys Leu Ile
        755                 760                 765

Ser Leu Pro Thr Ser Lys Asn Trp Thr Phe Gly Pro Gln Asp Val Asp
    770                 775                 780

Glu Leu Ile Phe Met Leu Ser Asp Ser Pro Gly Val Met Cys Arg Pro
785                 790                 795                 800

Ser Arg Val Lys Gln Met Phe Ala Ser Arg Ala Cys Arg Lys Ser Val
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805 810 815

Met Ile Gly Thr Ala Leu Asn Thr Ser Glu Met Lys Lys Leu Ile Thr
820 825 830

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850 855 860

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Phe Asp Lys Ile Glu Val Arg Asp Asn Gly Glu Gly Ile Lys Ala Val
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Asp Ala Pro Val Met Ala Met Lys Tyr Tyr Thr Ser Lys Ile Asn Ser

65 70 75 80

His Glu Asp Leu Glu Asn Leu Thr Thr Tyr Gly Phe Arg Gly Glu Ala
85 90 95

Leu Gly Ser Ile Cys Cys Ile Ala Glu Val Leu Ile Thr Thr Arg Thr
100 105 110

Ala Ala Asp Asn Phe Ser Thr Gln Tyr Val Leu Asp Gly Ser Gly His
115 120 125

Ile Leu Ser Gln Lys Pro Ser His Leu Gly Gln Gly Thr Thr Val Thr
130 135 140

Ala Leu Arg Leu Phe Lys Asn Leu Pro Val Arg Lys Gln Phe Tyr Ser
145 150 155 160

Thr Ala Lys Lys Cys Lys Asp Glu Ile Lys Lys Ile Gln Asp Leu Leu
165 170 175

Met Ser Phe Gly Ile Leu Lys Pro Asp Leu Arg Ile Val Phe Val His
180 185 190

Asn Lys Ala Val Ile Trp Gln Lys Ser Arg Val Ser Asp His Lys Met
195 200 205

Ala Leu Met Ser Val Leu Gly Thr Ala Val Met Asn Asn Met Glu Ser
210 215 220

Phe Gln Tyr His Ser Glu Glu Ser Gln Ile Tyr Leu Ser Gly Phe Leu
225 230 235 240

Pro Lys Cys Asp Ala Asp His Ser Phe Thr Ser Leu Ser Thr Pro Glu
245 250 255

Arg Ser Phe Ile Phe Ile Asn Ser Arg Pro Val His Gln Lys Asp Ile
260 265 270

Leu Lys Leu Ile Arg His His Tyr Asn Leu Lys Cys Leu Lys Glu Ser
275 280 285

Thr Arg Leu Tyr Pro Val Phe Phe Leu Lys Ile Asp Val Pro Thr Ala
290 295 300

Asp Val Asp Val Asn Leu Thr Pro Asp Lys Ser Gln Val Leu Leu Gln
305 310 315 320

Asn Lys Glu Ser Val Leu Ile Ala Leu Glu Asn Leu Met Thr Thr Cys
325 330 335

Tyr Gly Pro Leu Pro Ser Thr Asn Ser Tyr Glu Asn Asn Lys Thr Asp
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Val Ser Ala Ala Asp Ile Val Leu Ser Lys Thr Ala Glu Thr Asp Val
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Leu Phe Asn Lys Val Glu Ser Ser Gly Lys Asn Tyr Ser Asn Val Asp
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Thr Ser Val Ile Pro Phe Gln Asn Asp Met His Asn Asp Glu Ser Gly
385 390 395 400

Lys Asn Thr Asp Asp Cys Leu Asn His Gln Ile Ser Ile Gly Asp Phe
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Gly Tyr Gly His Cys Ser Ser Glu Ile Ser Asn Ile Asp Lys Asn Thr
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Lys Asn Ala Phe Gln Asp Ile Ser Met Ser Asn Val Ser Trp Glu Asn
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Ser Gln Thr Glu Tyr Ser Lys Thr Cys Phe Ile Ser Ser Val Lys His
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Thr Gln Ser Glu Asn Gly Asn Lys Asp His Ile Asp Glu Ser Gly Glu
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Asn Glu Glu Glu Ala Gly Leu Glu Asn Ser Ser Glu Ile Ser Ala Asp
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Glu Trp Ser Arg Gly Asn Ile Leu Lys Asn Ser Val Gly Glu Asn Ile
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Glu Pro Val Lys Ile Leu Val Pro Glu Lys Ser Leu Pro Cys Lys Val
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Ser Cys Asn Lys Lys Ser Asn Val Ile Asp Asn Lys Ser Gly Lys Val
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Thr Ala Tyr Asp Leu Leu Ser Asn Arg Val Ile Lys Lys Pro Met Ser
565 570 575

Ala Ser Ala Leu Phe Val Gln Asp His Arg Pro Gln Phe Leu Ile Glu
580 585 590

Asn Pro Lys Thr Ser Leu Glu Asp Ala Thr Leu Gln Ile Glu Glu Leu
595 600 605

Trp Lys Thr Leu Ser Glu Glu Glu Lys Leu Lys Tyr Glu Glu Lys Ala
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Thr Lys Asp Leu Glu Arg Tyr Asn Ser Gln Met Lys Arg Ala Ile Glu

625 630 635 640

Gln Glu Ser Gln Met Ser Leu Lys Asp Gly Arg Lys Lys Ile Lys Pro
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Thr Ser Ala Trp Asn Leu Ala Gln Lys His Lys Leu Lys Thr Ser Leu
660 665 670

Ser Asn Gln Pro Lys Leu Asp Glu Leu Leu Gln Ser Gln Ile Glu Lys
675 680 685

Arg Arg Ser Gln Asn Ile Lys Met Val Gln Ile Pro Phe Ser Met Lys
690 695 700

Asn Leu Lys Ile Asn Phe Lys Lys Gln Asn Lys Val Asp Leu Glu Glu
705 710 715 720

Lys Asp Glu Pro Cys Leu Ile His Asn Leu Arg Phe Pro Asp Ala Trp
725 730 735

Leu Met Thr Ser Lys Thr Glu Val Met Leu Leu Asn Pro Tyr Arg Val
740 745 750

Glu Glu Ala Leu Leu Phe Lys Arg Leu Leu Glu Asn His Lys Leu Pro
755 760 765

Ala Glu Pro Leu Glu Lys Pro Ile Met Leu Thr Glu Ser Leu Phe Asn
770 775 780

Gly Ser His Tyr Leu Asp Val Leu Tyr Lys Met Thr Ala Asp Asp Gln
785 790 795 800

Arg Tyr Ser Gly Ser Thr Tyr Leu Ser Asp Pro Arg Leu Thr Ala Asn
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Gly Phe Lys Ile Lys Leu Ile Pro Gly Val Ser Ile Thr Glu Asn Tyr
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Leu Glu Ile Glu Gly Met Ala Asn Cys Leu Pro Phe Tyr Gly Val Ala
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Asp Leu Lys Glu Ile Leu Asn Ala Ile Leu Asn Arg Asn Ala Lys Glu
850 855 860

Val Tyr Glu Cys Arg Pro Arg Lys Val Ile Ser Tyr Leu Glu Gly Glu
865 870 875 880

Ala Val Arg Leu Ser Arg Gln Leu Pro Met Tyr Leu Ser Lys Glu Asp
885 890 895

Ile Gln Asp Ile Ile Tyr Arg Met Lys His Gln Phe Gly Asn Glu Ile
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Pro Glu Thr Thr
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aac 3063

<210> 11
<211> 934
<212> PRT
<213> Homo sapiens

<400> 11

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20 25 30

Thr Val Arg Leu Phe Asp Arg Gly Asp Phe Tyr Thr Ala His Gly Glu
35 40 45

Asp Ala Leu Leu Ala Ala Arg Glu Val Phe Lys Thr Gln Gly Val Ile

50 55 60

Lys Tyr Met Gly Pro Ala Gly Ala Lys Asn Leu Gln Ser Val Val Leu
65 70 75 80

Ser Lys Met Asn Phe Glu Ser Phe Val Lys Asp Leu Leu Leu Val Arg
85 90 95

Gln Tyr Arg Val Glu Val Tyr Lys Asn Arg Ala Gly Asn Lys Ala Ser
100 105 110

Lys Glu Asn Asp Trp Tyr Leu Ala Tyr Lys Ala Ser Pro Gly Asn Leu
115 120 125

Ser Gln Phe Glu Asp Ile Leu Phe Gly Asn Asn Asp Met Ser Ala Ser
130 135 140

Ile Gly Val Val Gly Val Lys Met Ser Ala Val Asp Gly Gln Arg Gln
145 150 155 160

Val Gly Val Gly Tyr Val Asp Ser Ile Gln Arg Lys Leu Gly Leu Cys
165 170 175

Glu Phe Pro Asp Asn Asp Gln Phe Ser Asn Leu Glu Ala Leu Leu Ile
180 185 190

Gln Ile Gly Pro Lys Glu Cys Val Leu Pro Gly Gly Glu Thr Ala Gly
195 200 205

Asp Met Gly Lys Leu Arg Gln Ile Ile Gln Arg Gly Gly Ile Leu Ile
210 215 220

Thr Glu Arg Lys Lys Ala Asp Phe Ser Thr Lys Asp Ile Tyr Gln Asp
225 230 235 240

Leu Asn Arg Leu Leu Lys Gly Lys Lys Gly Glu Gln Met Asn Ser Ala
245 250 255

Val Leu Pro Glu Met Glu Asn Gln Val Ala Val Ser Ser Leu Ser Ala
260 265 270

Val Ile Lys Phe Leu Glu Leu Leu Ser Asp Asp Ser Asn Phe Gly Gln
275 280 285

Phe Glu Leu Thr Thr Phe Asp Phe Ser Gln Tyr Met Lys Leu Asp Ile
290 295 300

Ala Ala Val Arg Ala Leu Asn Leu Phe Gln Gly Ser Val Glu Asp Thr
305 310 315 320

Thr Gly Ser Gln Ser Leu Ala Ala Leu Leu Asn Lys Cys Lys Thr Pro
325 330 335

Gln Gly Gln Arg Leu Val Asn Gln Trp Ile Lys Gln Pro Leu Met Asp
340 345 350

Lys Asn Arg Ile Glu Glu Arg Leu Asn Leu Val Glu Ala Phe Val Glu
355 360 365

Asp Ala Glu Leu Arg Gln Thr Leu Gln Glu Asp Leu Leu Arg Arg Phe
370 375 380

Pro Asp Leu Asn Arg Leu Ala Lys Lys Phe Gln Arg Gln Ala Ala Asn
385 390 395 400

Leu Gln Asp Cys Tyr Arg Leu Tyr Gln Gly Ile Asn Gln Leu Pro Asn
405 410 415

Val Ile Gln Ala Leu Glu Lys His Glu Gly Lys His Gln Lys Leu Leu
420 425 430

Leu Ala Val Phe Val Thr Pro Leu Thr Asp Leu Arg Ser Asp Phe Ser
435 440 445

Lys Phe Gln Glu Met Ile Glu Thr Thr Leu Asp Met Asp Gln Val Glu
450 455 460

Asn His Glu Phe Leu Val Lys Pro Ser Phe Asp Pro Asn Leu Ser Glu
465 470 475 480

Leu Arg Glu Ile Met Asn Asp Leu Glu Lys Lys Met Gln Ser Thr Leu
485 490 495

Ile Ser Ala Ala Arg Asp Leu Gly Leu Asp Pro Gly Lys Gln Ile Lys
500 505 510

Leu Asp Ser Ser Ala Gln Phe Gly Tyr Tyr Phe Arg Val Thr Cys Lys
515 520 525

Glu Glu Lys Val Leu Arg Asn Asn Lys Asn Phe Ser Thr Val Asp Ile
530 535 540

Gln Lys Asn Gly Val Lys Phe Thr Asn Ser Lys Leu Thr Ser Leu Asn
545 550 555 560

Glu Glu Tyr Thr Lys Asn Lys Thr Glu Tyr Glu Glu Ala Gln Asp Ala
565 570 575

Ile Val Lys Glu Ile Val Asn Ile Ser Ser Gly Tyr Val Glu Pro Met
580 585 590

Gln Thr Leu Asn Asp Val Leu Ala Gln Leu Asp Ala Val Val Ser Phe
595 600 605

Ala His Val Ser Asn Gly Ala Pro Val Pro Tyr Val Arg Pro Ala Ile

610 615 620

Leu Glu Lys Gly Gln Gly Arg Ile Ile Leu Lys Ala Ser Arg His Ala
625 630 635 640

Cys Val Glu Val Gln Asp Glu Ile Ala Phe Ile Pro Asn Asp Val Tyr
645 650 655

Phe Glu Lys Asp Lys Gln Met Phe His Ile Ile Thr Gly Pro Asn Met
660 665 670

Gly Gly Lys Ser Thr Tyr Ile Arg Gln Thr Gly Val Ile Val Leu Met
675 680 685

Ala Gln Ile Gly Cys Phe Val Pro Cys Glu Ser Ala Glu Val Ser Ile
690 695 700

Val Asp Cys Ile Leu Ala Arg Val Gly Ala Gly Asp Ser Gln Leu Lys
705 710 715 720

Gly Val Ser Thr Phe Met Ala Glu Met Leu Glu Thr Ala Ser Ile Leu
725 730 735

Arg Ser Ala Thr Lys Asp Ser Leu Ile Ile Ile Asp Glu Leu Gly Arg
740 745 750

Gly Thr Ser Thr Tyr Asp Gly Phe Gly Leu Ala Trp Ala Ile Ser Glu
755 760 765

Tyr Ile Ala Thr Lys Ile Gly Ala Phe Cys Met Phe Ala Thr His Phe
770 775 780

His Glu Leu Thr Ala Leu Ala Asn Gln Ile Pro Thr Val Asn Asn Leu
785 790 795 800

His Val Thr Ala Leu Thr Thr Glu Glu Thr Leu Thr Met Leu Tyr Gln
805 810 815

Val Lys Lys Gly Val Cys Asp Gln Ser Phe Gly Ile His Val Ala Glu
820 825 830

Leu Ala Asn Phe Pro Lys His Val Ile Glu Cys Ala Lys Gln Lys Ala
835 840 845

Leu Glu Leu Glu Glu Phe Gln Tyr Ile Gly Glu Ser Gln Gly Tyr Asp
850 855 860

Ile Met Glu Pro Ala Ala Lys Lys Cys Tyr Leu Glu Arg Glu Gln Gly
865 870 875 880

Glu Lys Ile Ile Gln Glu Phe Leu Ser Lys Val Lys Gln Met Pro Phe
885 890 895

Thr Glu Met Ser Glu Glu Asn Ile Thr Ile Lys Leu Lys Gln Leu Lys
900 905 910

Ala Glu Val Ile Ala Lys Asn Asn Ser Phe Val Asn Glu Ile Ile Ser
915 920 925

Arg Ile Lys Val Thr Thr
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<212> DNA
<213> Homo sapiens

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gcttcgtgcg cttctttcag ggcatgccgg agaagccgac caccacagtg cgccttttcg 180

accggggcga cttctatacg gcgcacggcg aggacgcgct gctggccgcc cgggaggtgt 240

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atttggcata taaggcttct cctggcaatc tctctcagtt tgaagacatt ctctttggta 480

acaatgatat gtcagcttcc attggtgttg tgggtgttaa aatgtccgca gttgatggcc 540

agagacaggt tggagttggg tatgtggatt ccatacagag gaaactagga ctgtgtgaat 600

tccctgataa tgatcagttc tccaatcttg aggctctcct catccagatt ggaccaaagg 660

aatgtgtttt acccggagga gagactgctg gagacatggg gaaactgaga cagataattc 720

aaagaggagg aattctgatc acagaaagaa aaaaagctga cttttccaca aaagacattt 780

atcaggacct caaccggttg ttgaaaggca aaaagggaga gcagatgaat agtgctgtat 840

tgccagaaat ggagaatcag gttgcagttt catcactgtc tgcggtaatc aagtttttag 900

aactcttatc agatgattcc aactttggac agtttgaact gactactttt gacttcagcc 960

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gacaaagact tgttaaccag tggattaagc agcctctcat ggataagaac agaatagagg 1140

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aagatttact tcgtcgattc ccagatctta accgacttgc caagaagttt caaagacaag 1260

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actttagtac tgtagatatc cagaagaatg gtgttaaatt taccaacagc aaattgactt 1740

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atatttagta atattttact ttgaggacat tttcaaagat ttttattttg aaaaatgaga 3060

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ataaataaaa tcatgtagtt tgtgg 3145

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<211> 756
<212> PRT
<213> Homo sapiens

<400> 13

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Asn Arg Ile Ala Ala Gly Glu Val Ile Gln Arg Pro Ala Asn Ala Ile
20 25 30

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        35                  40                  45

Val Ile Val Lys Glu Gly Gly Leu Lys Leu Ile Gln Ile Gln Asp Asn
    50                  55                  60

Gly Thr Gly Ile Arg Lys Glu Asp Leu Asp Ile Val Cys Glu Arg Phe
65                  70                  75                  80

Thr Thr Ser Lys Leu Gln Ser Phe Glu Asp Leu Ala Ser Ile Ser Thr
                85                  90                  95

Tyr Gly Phe Arg Gly Glu Ala Leu Ala Ser Ile Ser His Val Ala His
            100                 105                 110

Val Thr Ile Thr Thr Lys Thr Ala Asp Gly Lys Cys Ala Tyr Arg Ala
        115                 120                 125

Ser Tyr Ser Asp Gly Lys Leu Lys Ala Pro Pro Lys Pro Cys Ala Gly
    130                 135                 140

Asn Gln Gly Thr Gln Ile Thr Val Glu Asp Leu Phe Tyr Asn Ile Ala
145                 150                 155                 160

Thr Arg Arg Lys Ala Leu Lys Asn Pro Ser Glu Glu Tyr Gly Lys Ile
                165                 170                 175

Leu Glu Val Val Gly Arg Tyr Ser Val His Asn Ala Gly Ile Ser Phe
            180                 185                 190

Ser Val Lys Lys Gln Gly Glu Thr Val Ala Asp Val Arg Thr Leu Pro
        195                 200                 205

Asn Ala Ser Thr Val Asp Asn Ile Arg Ser Ile Phe Gly Asn Ala Val
    210                 215                 220

Ser Arg Glu Leu Ile Glu Ile Gly Cys Glu Asp Lys Thr Leu Ala Phe
225                 230                 235                 240

Lys Met Asn Gly Tyr Ile Ser Asn Ala Asn Tyr Ser Val Lys Lys Cys
                245                 250                 255

Ile Phe Leu Leu Phe Ile Asn His Arg Leu Val Glu Ser Thr Ser Leu
            260                 265                 270

Arg Lys Ala Ile Glu Thr Val Tyr Ala Ala Tyr Leu Pro Lys Asn Thr
        275                 280                 285

His Pro Phe Leu Tyr Leu Ser Leu Glu Ile Ser Pro Gln Asn Val Asp
    290                 295                 300

Val Asn Val His Pro Thr Lys His Glu Val His Phe Leu His Glu Glu
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305 310 315 320

Ser Ile Leu Glu Arg Val Gln Gln His Ile Glu Ser Lys Leu Leu Gly
325 330 335

Ser Asn Ser Ser Arg Met Tyr Phe Thr Gln Thr Leu Leu Pro Gly Leu
340 345 350

Ala Gly Pro Ser Gly Glu Met Val Lys Ser Thr Thr Ser Leu Thr Ser
355 360 365

Ser Ser Thr Ser Gly Ser Ser Asp Lys Val Tyr Ala His Gln Met Val
370 375 380

Arg Thr Asp Ser Arg Glu Gln Lys Leu Asp Ala Phe Leu Gln Pro Leu
385 390 395 400

Ser Lys Pro Leu Ser Ser Gln Pro Gln Ala Ile Val Thr Glu Asp Lys
405 410 415

Thr Asp Ile Ser Ser Gly Arg Ala Arg Gln Gln Asp Glu Glu Met Leu
420 425 430

Glu Leu Pro Ala Pro Ala Glu Val Ala Ala Lys Asn Gln Ser Leu Glu
435 440 445

Gly Asp Thr Thr Lys Gly Thr Ser Glu Met Ser Glu Lys Arg Gly Pro
450 455 460

Thr Ser Ser Asn Pro Arg Lys Arg His Arg Glu Asp Ser Asp Val Glu
465 470 475 480

Met Val Glu Asp Asp Ser Arg Lys Glu Met Thr Ala Ala Cys Thr Pro
485 490 495

Arg Arg Arg Ile Ile Asn Leu Thr Ser Val Leu Ser Leu Gln Glu Glu
500 505 510

Ile Asn Glu Gln Gly His Glu Val Leu Arg Glu Met Leu His Asn His
515 520 525

Ser Phe Val Gly Cys Val Asn Pro Gln Trp Ala Leu Ala Gln His Gln
530 535 540

Thr Lys Leu Tyr Leu Leu Asn Thr Thr Lys Leu Ser Glu Glu Leu Phe
545 550 555 560

Tyr Gln Ile Leu Ile Tyr Asp Phe Ala Asn Phe Gly Val Leu Arg Leu
565 570 575

Ser Glu Pro Ala Pro Leu Phe Asp Leu Ala Met Leu Ala Leu Asp Ser
580 585 590

Pro Glu Ser Gly Trp Thr Glu Glu Asp Gly Pro Lys Glu Gly Leu Ala
595 600 605

Glu Tyr Ile Val Glu Phe Leu Lys Lys Lys Ala Glu Met Leu Ala Asp
610 615 620

Tyr Phe Ser Leu Glu Ile Asp Glu Glu Gly Asn Leu Ile Gly Leu Pro
625 630 635 640

Leu Leu Ile Asp Asn Tyr Val Pro Pro Leu Glu Gly Leu Pro Ile Phe
645 650 655

Ile Leu Arg Leu Ala Thr Glu Val Asn Trp Asp Glu Glu Lys Glu Cys
660 665 670

Phe Glu Ser Leu Ser Lys Glu Cys Ala Met Phe Tyr Ser Ile Arg Lys
675 680 685

Gln Tyr Ile Ser Glu Glu Ser Thr Leu Ser Gly Gln Gln Ser Glu Val
690 695 700

Pro Gly Ser Ile Pro Asn Ser Trp Lys Trp Thr Val Glu His Ile Val
705 710 715 720

Tyr Lys Ala Leu Arg Ser His Ile Leu Pro Pro Lys His Phe Thr Glu
725 730 735

Asp Gly Asn Ile Leu Gln Leu Ala Asn Leu Pro Asp Leu Tyr Lys Val
740 745 750

Phe Glu Arg Cys
755

<210> 14
<211> 2484
<212> DNA
<213> Homo sapiens

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gagatgattg agaactgttt agatgcaaaa tccacaagta ttcaagtgat tgttaaagag 180

ggaggcctga agttgattca gatccaagac aatggcaccg ggatcaggaa agaagatctg 240

gatattgtat gtgaaaggtt cactactagt aaactgcagt cctttgagga tttagccagt 300

atttctacct atggctttcg aggtgaggct ttggccagca taagccatgt ggctcatgtt 360

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aaactgaaag cccctcctaa accatgtgct ggcaatcaag ggacccagat cacggtggag 480

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<211> 133
<212> PRT
<213> Homo sapiens

<400> 15

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Pro Ile Asp Arg Lys Ser Val His Gln Ile Cys Ser Gly Gln Val Val
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Leu Ser Leu Ser Thr Ala Val Lys Glu Leu Val Glu Asn Ser Leu Asp
35 40 45

Ala Gly Ala Thr Asn Ile Asp Leu Lys Leu Lys Asp Tyr Gly Val Asp
50 55 60

Leu Ile Glu Val Ser Asp Asn Gly Cys Gly Val Glu Glu Glu Asn Phe
65 70 75 80

Glu Gly Leu Thr Leu Lys His His Thr Ser Lys Ile Gln Glu Phe Ala
85 90 95

Asp Leu Thr Gln Val Glu Thr Phe Gly Phe Arg Gly Glu Ala Leu Ser
100 105 110

Ser Leu Cys Ala Leu Ser Asp Val Thr Ile Ser Thr Cys His Ala Ser
115 120 125

Ala Lys Val Gly Thr
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<210> 16
<211> 426
<212> DNA
<213> Homo sapiens

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ctgagtctaa gcactgcggt aaaggagtta gtagaaaaca gtctggatgc tggtgccact 180

aatattgatc taaagcttaa ggactatgga gtggatctta ttgaagtttc agacaatgga 240

tgtggggtag aagaagaaaa cttcgaaggc ttaactctga aacatcacac atctaagatt 300

caagagtttg ccgacctaac tcaggttgaa acttttggct ttcgggggga agctctgagc 360

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acttga 426

<210> 17
<211> 70
<212> PRT
<213> Homo sapiens

<400> 17

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Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr Gly Met Asn
20 25 30

Trp Val Lys Gln Ala Pro Gly Lys Gly Leu Lys Trp Met Gly Trp Ile
35 40 45

Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Asp Asp Phe Lys Gly Arg
50 55 60

Phe Ala Phe Ser Leu Glu
65 70

<210> 18
<211> 70
<212> PRT
<213> Homo sapiens

<400> 18

Leu Gln Gln Ser Gly Ala Glu Leu Val Arg Pro Gly Thr Ser Val Lys
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Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr Gly Met Asn
20 25 30

Trp Val Lys Gln Ala Pro Gly Lys Gly Leu Lys Trp Met Gly Trp Ile
35 40 45

Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Asp Asp Phe Lys Gly Arg
50 55 60

Phe Ala Phe Ser Leu Glu
65 70

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<212> PRT
<213> Artificial

<220>
<223> Consensus sequence

<400> 19

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20 25 30

Gln Ala Pro Gly Lys Gly Leu Lys Trp Met Gly Trp Ile Asn Thr Tyr
35 40 45

Thr Gly Glu Pro Thr Tyr Ala Asp Asp Phe Lys Gly Arg Phe Ala Phe
50 55 60

Ser Leu Glu
65

<210> 20
<211> 45
<212> PRT
<213> Homo sapiens

<400> 20

Ser Ala Ser Ser Ser Val Ser Ser Ser Tyr Phe His Trp Tyr Gln Gln
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Lys Ser Gly Ala Ser Pro Lys Pro Leu Ile His Arg Thr Ser Asn Leu
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Ala Ser Gly Val Pro Ala Arg Phe Ser Gly Ser Gly Ser
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Ser Ala Ser Ser Ser Val Ser Ser Ser Tyr Phe His Trp Tyr Gln Gln
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Ala Ser Gly Val Pro Ala Arg Phe Ser Gly Ser Gly Ser
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— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ANTIBODIES AND METHODS FOR GENERATING GENETICALLY ALTERED ANTIBODIES WITH HIGH AFFINITY

(57) Abstract: Dominant negative alleles of human mismatch repair genes can be used to generate hypermutable cells and organisms. By introducing these genes into cells and transgenic animals, new cell lines and animal varieties with novel and useful properties can be prepared more efficiently than by relying on the natural rate of mutation. These methods are useful for generating genetic diversity within immunoglobulins genes directed against an antigen of interest to produce altered antibodies with enhanced biochemical activity. Moreover, these methods are useful for generating antibody-producing cells with increased level of antibody production. The invention also provides methods for increasing the affinity of monoclonal antibodies and monoclonal antibodies with increased affinity.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/28722

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C12Q 1/68; C07K 16/00
US CL : 435/6; 530/387.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6; 530/387.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LOW NM, et al. Mimicking somatic hypermutation: affinity maturation of anitbodies displayed on bacteriophage using a bacterial mutator strain. J. Mol. Biol. 1996, Vol. 260, pages 359-368; see entire document.	1-15
Y	COIA G, et al. Protein affinity maturation in vivo using E. coli mutator cells. J. Immunol. Methods. 2001, Vol. 251, pages 187-193; see entire document.	1-15
Y	WABL M, et al. Hypermutation in antibody affinity maturation. Curr. Opin. Immunol. 1999, Vol. 11, pages 186-189; see entire document.	1-15
Y	LI Y, et al. Three-dimensional structures of the free and antigen-bound Fab from monoclonal antilysozyme antibody HyHEL-63. Biochemistry. May 2000, Vol. 39, pages 6296-6309; see entire document.	1-15

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner of Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 305-3230	Authorized officer Stephen L. Rawlings Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

PCT/US03/28722

Continuation of B. FIELDS SEARCHED Item 3:
WEST, MEDLINE, GENESEQ, GENEMBL, PUBLISHED APPLICATIONS, ISSUED PATENTS, UNIPROT: SEQ ID NOS: 18 AND 21; affinity maturation; antibody; mutator; hypermutation; framework